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Extended abstract (for 3 years of research)

ErbB-2, also called HER2 or Neu, is a marker of aggressive breast cancers and it can predict short patient survival and resistance to conventional chemotherapy. Antibodies directed to ErbB-2 (Herceptin®) are already used to treat metastasizing breast tumors. Despite the wealth of basic and clinical information on ErbB-2, the exact molecular mechanisms underlying its action in human cancers remained unknown. Specifically, because ErbB-2 is structurally and functionally related to well-characterized receptors for growth factors like the epidermal growth factor (EGF) and neuregulins (NRGs), it has been assumed that ErbB-2 acts as a direct receptor for a putative ligand. On the contrary, we have tested with the help of the IDEA grant an alternative hypothesis, namely: that ErbB-2 acts as a common low affinity receptor for many stromal ligands by binding to their putative low affinity sites. In other words, we predicted that all ErbB ligands are bivalent, thereby ErbB-2 overexpression sensitizes tumor cells to the multiple growth factors by assisting their binding to the respective direct receptor. The following strategies were employed to test the validity of the model:

- (i) The prediction that ErbB-2 binds no direct ligand but it can assist binding of all ligands has been tested by analyzing the family of NRG2 ligands and epiregulin. In addition, we tested all representative ligands of pox viruses, and also discovered a new neuregulin that we called NRG4. All these analyses confirmed the prediction that no ligand binds with high affinity to ErbB-2, but binding of all ligands tested was assisted by the co-receptor.
- (ii) Comparison of signaling by two ligands, EGF and TGF α , revealed an important outcome of the bivalence model, namely: Some ligands may promote degradation of their receptors, while other ligands may direct their receptors to recycling because the ligand-receptor complexes break in an early endosomal compartment.
- (iii) By using biophysical measurements and soluble receptor mutants we obtained evidence for low affinity binding of ligands to ErbB-2. The putative binding site was tentatively mapped to the N-terminal portion of the receptor by using specific monoclonal antibodies.

- (iv) The last route of studies examined the bivalence hypothesis by synthesizing short peptides encompassing the two putative binding sites of NRG1. Such peptides retained weak, but specific, biological activity, in line with a bivalence mode of ligand-receptor interactions.

In conclusion, several lines of evidence support the bivalence model of ligand-receptor interactions and collectively attribute to ErbB-2 a role as a co-receptor. Future studies will further test the model by attempting crystalization of ErbB-2 in a complex with a ligand and a direct receptor.

Introduction

The ErbB (also called HER) family of receptors includes four transmembrane glycoproteins that embody tyrosine-specific kinase domains in their intracellular portions. All receptors, but ErbB-3 (Guy et al., 1994), are active kinases that autophosphorylate, or trans-phosphorylate, other substrates upon binding of a ligand to the extracellularly-located binding sites. The mammalian ligands of ErbBs are synthesized as transmembrane precursors, whose ectodomains include a variety of structural motifs, but they all share a 50-60 amino acid-long epidermal growth factor-(EGF-) like motif of six cysteine residues [reviewed in (Burden and Yarden, 1997)]. This module is essential and sufficient for ErbB binding. Thus, whereas ErbB-1 binds several ligands, EGF and the transforming growth factor α (TGF α) being the prototypes, at least four distinct types of neuregulins bind to ErbB-3 and/or ErbB-4. ErbB-2 binds no known ligand with high affinity, but it can act as a shared low-affinity receptor for many, if not all, ErbB ligands (Tzahar and Yarden, 1998). Targeted inactivation of genes encoding ErbBs or their ligands revealed their multiple essential functions in embryonic development. For example, ErbB-1 and some of its ligands are involved in growth and differentiation of certain types of epithelia (Miettinen et al., 1995), whereas neuregulins and their receptors are important for cardiac and neural development (Gassmann et al., 1995; Meyer and Birchmeier, 1995). Nerve regeneration after injury and healing of skin wounds are two examples of ErbB- and neuregulin-controlled processes during adult life (Danilenko et al., 1995). Likewise, neuregulins as well as several ErbB-1 ligands participate in the highly regulated process that control development of the mammary gland during pregnancy (Yang et al., 1995). Mutant forms of ErbBs and up-regulated expression of the wild type forms contribute to certain malignancies in animals and in human [reviewed in (Salomon et al., 1995)]. An example is the frequent overexpression of ErbB-2 in breast cancer and its association with poor prognosis (Slamon et al., 1989).

Signal transduction by all growth factor receptors is preceded by a ligand-induced receptor dimerization step, originally found with ErbB-1 (Yarden and

Schlessinger, 1987). Complexity at this step is exhibited by the ErbB family, as the four receptors can form all ten homo- and heterodimeric combinations (Cohen et al., 1996; Riese et al., 1995; Tzahar et al., 1996; Zhang et al., 1996). Within the dimer, the receptors undergo tyrosine phosphorylation at sites that function as reversible docking points for signaling proteins containing phosphotyrosine-binding motifs [reviewed in (van der Geer et al., 1994)]. Because each ErbB receptor is endowed with its own specific set of docking sites, the existence of many receptor dimers can diversify the output signal. Moreover, the specific dimers that will form depend not only on the identity of the stimulating ligand, but also on the relative level of expression of the interacting receptors, with clear preference for ErbB-2 as a dimer partner (Graus-Porta et al., 1997; Tzahar et al., 1996). How an ErbB-specific ligand causes rapid dimerization of its receptor is currently an open question, which is complicated by the absence of a duplicated structure in the EGF-like domain, or a dimeric ligand species. An interesting aspect related to the mechanism of ligand-induced receptor dimerization is the role played by ErbB-2: Although ErbB-2 binds no ligand with high affinity, it can be efficiently recruited into heterodimeric complexes. Most abundant is the ErbB-2/ErbB-3 complex, whose mitogenic (Pinkas-Kramarski et al., 1996) and oncogenic (Alimandi et al., 1995; Wallasch et al., 1995) activities exceed those of other receptor complexes. The ability of ErbB-2 to reconstitute an extremely potent receptor complex may explain the role of this oncoprotein in human cancer (Klapper et al., 1999). Thus, it is important to understand the exact mechanism by which an ErbB-2/ErbB-3 complex is generated. Our analysis of this process led us to propose that the ligand (neuregulin-1, or Neu differentiation factor- NDF) is bivalent (Tzahar et al., 1997). The basis for this proposition was the remarkable ability of ErbB-2 to enhance the binding affinity of other ErbBs, and especially ErbB-3 (Karunagaran et al., 1996; Peles et al., 1993; Sliwkowski et al., 1994). According to this sequential model, the ligand first binds with low affinity to ErbB-3, which presents it to a low affinity site on ErbB-2.

If ErbB ligands are indeed bivalent, their two binding sites may be identifiable through mutagenesis. However, such extensive analyses led to no conclusive evidence for bivalence (Groenen et al., 1994). The solution structure of the

EGF-like domain of NDF- β was recently solved to high resolution using NMR (Jacobsen et al., 1996; Nagata et al., 1994). The molecule contains two β sheets: the N-terminal domain comprises a three-stranded β -sheet, whereas the C-terminal part contains a smaller two-stranded sheet. Four hydrogen bonds stabilize the relative orientation of the two domains. Homologue scanning enabled mapping of the regions of the EGF-like domain that participate in receptor specificity determination (Barbacci et al., 1995; Harris et al., 1998). Based on these studies it appears likely that the most N-terminal three to five residues of the NDF- β EGF-like domain confer specificity to ErbB-3, whereas the specificity to ErbB-1 resides on a distinct domain. Alanine scanning mutagenesis confirmed the importance of the N- and C-termini of NDF, but it identified several more patches throughout the EGF-like domain that contribute to high binding affinity (Jones et al., 1998). The C-terminal portion of the EGF-like domains, although essential for high affinity binding, may not confer specificity to the exact ErbB (Harris et al., 1998). Consistent with this possibility, the α and β isoforms of NDF, which differ in this portion of the EGF-like domain, share specificity to ErbB-3 and to ErbB-4, but they significantly differ in binding affinity (Tzahar et al., 1994). Surprisingly, however, the two isoforms differ also qualitatively: only the β isoform can recruit ErbB-1 into dimers with ErbB-3 (Pinkas-Kramarski et al., 1996). In light of possible bivalence, these structure-function relationships may be interpreted in the following way (Tzahar et al., 1997): the N-terminus of the EGF-like domain (residues preceding the first cysteine) is the major site that determines specificity to ErbB-3, whereas the C-terminal region, including a portion of the C-loop and the linear sequence distal to the sixth cysteine, acts as a low affinity/broad specificity site which selects partners for dimerization.

We reasoned that correctness of the bivalence model of NDF would enable construction of a minimal analog capable of receptor binding and dimerization. The present study aimed at testing this possibility. The putative two binding sites of NDF were synthetically fused by using a linker whose length was inferred from the three-dimensional structure of the EGF-like domain. By testing several such analogs on cells

engineered to express specific ErbBs or their combinations, as well as on natural epithelial cells, we hereby provide evidence in support of a bivalence model.

Statement of Work: Current Status

Three lines of research were originally described in the research plan of the IDEA grant proposal. These were addressed and reported as follows:

- (i) Mapping of the putative low affinity/broad specificity binding site of ErbB-2: mutational analyses and construction of chimeric ErbB-1/ErbB-2 receptors tentatively mapped the binding site to the most N-terminal amino acids of ErbB-2. Part of the description of the specific experiments was detailed in previous reports. Our major technical obstacle was lack of membrane localization of deletion mutants encompassing portions of the extracellular domain of ErbB-2. However, intracellular retention of the mutants nevertheless enabled analysis of interactions with a set of mAbs. On the basis of this analysis we concluded that mAb capable of displacing EGF and NDF interact with the N-terminus of ErbB-2. In the next step this portion of ErbB-2 was implanted into ErbB-1. Currently we test EGF binding as well as mAb binding to the chimeric receptor, but our preliminary results are in line with the proposed mapping of the putative binding site.
- (ii) Biophysical and biochemical measurements of ligand binding to ErbB-2- Soluble ErbB-2 molecules were constructed as fusions with the Fc portion of human IgG1. By using Biacore analyses we obtained evidence for direct interactions between EGF and NDF and the soluble receptor. The measured affinity constants (K_D) were in the micromolar range. Importantly, comparison with soluble ErbB-3 and ErbB-1 indicated that the dissociation constants of ErbB-2 are remarkably more rapid than those of the two receptors. These results were described in our previous IDEA reports and also in a publication in the EMBO Journal (E. Tzahar et al. 1997).
- (iii) Identification of the two binding sites of NDF- The Body of the present report deals primarily with this specific aim (see below).

Along with these lines of research we attempted to generate effective blockers of ErbB signaling that are based upon a bivalence model. The following three lines of studies were described in the original Statement of Work:

(i) Monoclonal antibodies directed to the putative binding site of ErbB-2 have been generated and selected by their ability to inhibit, in part, ligand binding to (hetero-) dimeric receptor combinations. When tested in tumor-bearing mice the mAbs (type II antibodies according to our nomenclature) effectively inhibited tumor growth. The antibodies were used when trying to map the putative ligand binding site of ErbB-2 (see above).

(ii) Random peptide libraries were screened with a soluble ErbB-2 protein in an effort to isolate a peptide antagonist. We previously reported the results of our screening of several different libraries. No peptide could be confirmed upon repeated screen at moderate stringency. This failure concentrated our present efforts on a short peptide agonist, in the hope that limited modifications will turn it into an antagonist.

(iii) A membrane anchored ErbB-2 was constructed in an effort to generate a dominant negative mutant capable of heterodimerization. The recombinant protein carries a GPI signal attachment site which was derived from rat contactin. The protein was properly expressed at the cell surface and could be released by using phospholipase C. Furthermore, by using a radiolabeled NDF and covalent crosslinking reagents we detected heterodimers between ErbB-3 and the short mutant of ErbB-2. Quantitative comparison with a kinase-defective mutant of ErbB-2 suggests that the heterodimerizing capacity of the GPI-ErbB-2 fusion protein was relatively compromised. Currently we investigate the possibility that the kinase domain of ErbB-2 stabilizes dimers, and therefore deletion of this domain weakens heterodimers.

Body

Design of peptides carrying essential functional features of NDF- β

To construct potential analogs of the receptor binding portion of NDF- β we calculated the distance between the two putative receptor binding sites (Tzahar et al., 1997)

according to the predicted three-dimensional structure (Jacobsen et al., 1996). We then fitted the calculated distance, 15.9-16.6 Å, by a chemical linker moiety whose most extended conformation is similar in length. Figure 1 presents two amino caproic acid-(Aca-) based linkers we designed and their possible orientation relative to the two termini of NDF-β. The termini comprised the most distal five amino acids of each end of the EGF-like domain of NDF-β. The peptides were chemically synthesized, purified to homogeneity and their molecular weight confirmed by mass-spectrometry (see Materials and methods). The peptides we tested were denoted NF (to indicate linkage between the N- and C-termini of NDF) and NF', a similar molecule carrying a shorter linker (see Table 1). Several control peptides were also synthesized, including an analog (Ac-NF) whose length at extended conformation is only 9.6 Å, significantly less than predicted by our model (Fig. 1), an opposite-orientation peptide (FN, in which the N- and C-terminal amino acids of NDF were placed at the C- and N-terminus of the peptide, respectively) and a reverse-sequence peptide (Rev-FN). All peptides contained an amide bond at the C-terminus because this part is extended in the full length form of NDF.

NDF analogs are biologically active on engineered myeloid cells expressing the ErbB-2/ErbB-3 combination of receptors

To examine the hypothesis that the remote portions of the EGF-like domain of NDF-β are sufficient to reconstitute biological activity if placed in the correct distance from one another, we made use of a previously described series of myeloid cells (Pinkas-Kramarski et al., 1996; Shelly et al., 1998). Survival of the parental 32D myeloid cell line strictly depends on interleukin-3 (IL-3). However, introduction of certain ErbB proteins allows replacement of IL-3 with the cognate EGF-like ligand. Thus, D1 cells, which ectopically express ErbB-1, can be grown in the presence of EGF, whereas survival of D23 cells, expressing a combination of ErbB-2 and ErbB-3, may be supported by NDF. We first tested the ability of NDF and its various analogs to support short-term (24 hours) growth or survival of the 32D sublines. Cells singly expressing ErbB-1, ErbB-2, or ErbB-3 (D1, D2, and D3 cells, respectively) underwent no

proliferation in the presence of NDF- β , but cells singly expressing ErbB-4 (D4 cells) exhibited a moderate response to this ligand (Fig. 2, and data not shown). This observation was in line with our previous reports (Pinkas-Kramarski et al., 1996; Pinkas-Kramarski et al., 1996; Shelly et al., 1998; Tzahar et al., 1998), as was the ability of NDF- β to support growth of cells co-expressing ErbB-2 and ErbB-3 (Fig. 2, NDF panel). As predicted, the short synthetic compound NF was active on D23 cells (NF panel in Fig. 1). However, it displayed strict specificity to this heterodimeric combination; not only cells singly expressing each of the four ErbB proteins were not responsive, the combinations ErbB-1/ErbB-2 and ErbB-2/ErbB-4 showed no biological response (see below). Specificity of NF was implied also by the observation that neither a shorter analog (Ac-NF) nor a reverse version of NF could mimic its effect on D23 cells (Fig. 2).

The next set of experiments tested the long-term survival or proliferation effect of the various NDF analogs by following cell cultures during a three day-long period of time. These experiments not only confirmed that the combination of ErbB-2 with ErbB-3 was the only receptor pair that produced growth signals in response to NF, but they showed that another analog, NF', was almost as active as NF, and both factors shared strict specificity to the D23 cells (Fig. 3A). Although the extent of proliferative responses displayed by the D23 cells to various ligands displayed some variation between experiments, in no experiment was the effect of NF or NF' similar in intensity to that of NDF. The latter ligand was at least as potent as the ultimate growth factor of 32D cells, namely: IL-3. Like in the short-term assay of cell growth, no analog of NDF, other than NF and NF', was active on D23 or any other cell line we tested, including ErbB-1-containing complexes (Fig. 3B, and data not shown).

Next, we wished to analyze whether the biological effects elicited by NF and NF' were funneled through the activation of a canonical NDF signaling pathway. To this end we firstly checked receptor tyrosine phosphorylation and secondly stimulation of the mitogen-activated protein kinase (MAPK), a common target of all ErbB combinations (Pinkas-Kramarski et al., 1996). Cultures of the various 32D sublines were shortly incubated with NDF or its analogs, whole lysates prepared and resolved by gel

electrophoresis. Western blots were probed with either anti-phosphotyrosine antibodies, or with a murine monoclonal antibody (mAb) specific to the activated, doubly phosphorylated form of MAPK/Erk (Yung et al., 1997). As expected, stimulation with NDF increased tyrosine phosphorylation of a 190 kDa protein in D23 cells, but exposure to NF or NF' was less effective (Fig. 4). No other analog of NDF was active in this assay. Likewise, NDF potently stimulated activation of the two forms of Erk, but NF and NF' were the only analogs that enhanced phosphorylation, albeit with weaker potency, of these two MAPK proteins. In experiments that are not presented we found that no other sub-line of the 32D cells we used responded to NDF analogs by increased phosphorylation of either ErbB or MAPK. Taken together, the results presented in Figures 2-4 indicate that certain NDF analogs retain, to some extent, the ability to stimulate in a specific manner the ErbB-2/ErbB-3 heterodimer when the component receptors are co-expressed in a model myeloid cell system.

Short NDF analogs are mitogenic on ErbB-expressing epithelial cells

Because ErbB proteins are rarely expressed in myeloid cells such as the 32D cells, we tested the ability of the short NDF analogs to stimulate proliferation of natural cell lines. Epithelial mammary cells derived from human tumors were chosen for this purpose because the parental tissue is a well characterized physiological target of neuregulins (Yang et al., 1995). MDA-MB453 mammary cells co-overexpress ErbB-2 and ErbB-3, but no expression of ErbB-1 is detectable, and ErbB-4 levels are moderate (Plowman et al., 1993). Incubation of these cells in the absence of serum growth factors, but in the presence of NDF, resulted in a steady increase in cell numbers over the first four days in culture (Fig. 5A). Longer incubation intervals led to a gradual decrease in cell numbers and concomitant cell flattening and increased synthesis of neutral lipids (data not shown). Similar effects were previously observed with some ErbB-2-overexpressing cell lines (Bacus et al., 1993; Plowman et al., 1993; Plowman et al., 1993). Likewise, a four day-long treatment with either NF or NF' caused moderate growth signals that were not displayed by control cultures (Fig. 5A). By contrast, none of the other NDF analogs we synthesized was active on MDA-MB453 cells (Fig. 5B). Similar to factor-stimulated

D23 cells, growth stimulation was associated with rapid activation of the MAPK pathway: NDF, as well as its short analogs, stimulated rapid and extensive phosphorylation of Erk proteins in these mammary epithelial cells, but the response to the analogs was slightly delayed (Fig. 6A). That the stimulatory effect was not due to differences in protein content of the analyzed samples was confirmed by re-blotting with antibodies to the unmodified form of MAPK/Erk (Fig. 6A). Similar experiments were performed with rev-FN, Ac-NF, and the FN control peptides but no MAPK activation was observed (data not shown). To confirm MAPK activation by the short NDF analogs we also examined the sub-cellular localization of the active enzyme. In cells arrested at the G₀ phase of the cell cycle, p42 and p44 MAPK are mainly cytoplasmic, but upon stimulation by various extracellular ligands they translocate into the nucleus to trigger a mitogenic response (Lenormand et al., 1993). MDA-MB453 cells were growth-arrested by withdrawal of serum growth factors, and then they were treated for 5 minutes with NF or NF'. Staining with antibodies to the active form of the MAPK revealed that the two analogs, like the parental NDF molecule, induced the appearance of an active MAPK in the nuclei of treated cells, but practically no staining was observed with untreated cells (red stain in Fig. 6B). Co-staining with a DNA-specific dye (DAPI, blue staining in Fig. 6B) helped identify nuclei.

Unlike MDA-MB453 cells, which overexpress ErbB-2, only low level expression of this protein characterizes the MCF-7 breast cancer cell line. These cells exhibited growth stimulation in response to the two active analogs of NDF, with no late phase of cellular inhibition (Fig. 6 and data not shown). The photographs shown in Figure 7A depict cultures treated for three days with the indicated factors in the absence of serum. Evidently, extensive multiplication of MCF-7 cells was induced by the two short analogs of NDF. As was the case with D23 and MDA-MB453 cells, growth stimulation was accompanied by a rapid increase in MAPK activity (Fig. 7B). Control peptides like FN, Ac-NF and Rev-FN were unable to stimulate MAPK and cell growth (Fig. 7B and data not shown), consistent with their lack of function on other cell types. Sorting of MCF-7 cells was used to analyze cell cycle progression. This analysis revealed that the control peptides did not affect the fraction of cells undergoing

apoptosis in the absence of serum factors (approximately $28\pm 2\%$), but NDF as well as its short active analogs reduced this fraction (Fig. 7C). Characteristic cell cycle profiles observed with cultures treated with NF', or NDF as control, are shown in Figure 7D. We conclude that two analogs of NDF, NF and NF', can promote cell cycle progression of natural, as well as engineered ErbB-expressing cells.

The biologically active short analogs compete with NDF for cellular binding sites

Because NF and NF' stimulated 32D cells only when they expressed ErbB-2 and ErbB-3, and the biological effects were qualitatively similar to those exhibited by NDF, we predicted that the synthetic analog will share with NDF specific cellular binding sites. The radio-ligand competition assay presented in Figure 8 confirmed this supposition. To increase sensitivity, the ligand competition assay was performed with SKBr-3 breast cancer cells, which overexpress ErbB-2 in the presence of ErbB-3. Cells were incubated with a low concentration of a radiolabeled NDF- β in the presence of increasing concentrations of either unlabeled NDF or unlabeled short analogs. Evidently, the two analogs could compete with the radiolabeled tracer for specific cellular binding sites, but their potency was reduced by 10-fold or more relative to the full-length EGF-like domain of NDF (Fig. 8). In experiments that are not presented we repeated the ligand competition assay with a derivative of MCF-7 cell line that overexpresses ErbB-2 and obtained similar results. None of the biologically inactive analogs we tested was able to displace surface-bound NDF, and EGF was partially active only at very high concentrations (Fig. 8, and data not shown), consistent with its low affinity to an ErbB-2/ErbB-3 heterodimer (Alimandi et al., 1997; Pinkas-Kramarski et al., 1998). Thus, the relative displacement efficiency of short synthetic analogs confirms their ErbB specificity and can explain their compromised biological activity relative to the parental NDF molecule.

Materials and Methods

Materials

EGF (human, recombinant) was purchased from Sigma (St. Louis, MO). Recombinant human NDF β ₁₁₇₇₋₂₄₆ (NRG1- β 1) was obtained from Amgen (Thousand Oaks, CA). Radioactive materials were purchased from Amersham (Buckinghamshire, United Kingdom). IODOGEN was from Pierce. Polyclonal rabbit anti-Erk2 (C-14) antiserum and a monoclonal anti-phosphotyrosine antibody (PY-20) were purchased from Santa-Cruz Biotechnology (Santa-Cruz, CA). A polyclonal antibody against the C-terminal portion of ErbB-2 was generated as described (Peles et al., 1991). A murine monoclonal antibody to an active form of MAPK/Erk (doubly phosphorylated on both tyrosine and threonine residues of the TEY motif) was from Sigma (Yung et al., 1997). Binding buffer contained RPMI-1640 medium with 0.2 % bovine serum albumin (BSA) and 20 mM HEPES (pH 7.4). HNTG buffer contained 20 mM HEPES (pH7.5), 150 mM NaCl, 0.1 % Triton X-100 and 10% glycerol. Solubilization buffer contained 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1 % SDS, 1.5 mM EGTA, 1.5 mM MgCl₂, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (0.15 trypsin inhibitor unit/ ml) and 10 μ g/ml leupeptin.

Peptide synthesis

Peptides were synthesized on an Applied Biosystem (ABI) model 431 peptide synthesizer fortified with UV feedback monitoring at 301 nm, and using FMOC-Rink amide AM resin. The conventional ABI monitor-previous-peak-algorithm was employed up to five times with a cut-off of 3.5% of the first deprotection. A secondary deprotection (using 2% DPU/ 2 % piperidine /96 % NMP) was performed and followed by double coupling. Acetic anhydride/HOBT capping was utilized at the end of each coupling, followed by washing with 1:1 trifluoroethanol /DCM. The peptides were deprotected and removed from the resin as described (King et al., 1990), with the following modifications: methoxyndole (2%) was added to reagent K, and the reaction time was changed to 3.5 hours. Small quantities of the reduced peptides were purified

by reverse-phase HPLC and examined by matrix-assisted laser desorption ionization (MALDI) mass spectral analysis. The crude reduced proteins were dissolved in a Tris-HCl buffer (PH 6.0) containing guanidium-HCl (6M) and diluted to a concentration of 0.06 mg/ml in methionine-containing buffer (10 mM) that included 1.5 mM cystine, 0.75 mM cysteine, and 100 mM Tris (pH 8.0). The mixture was stirred for 48 hours at cold, and the oxidized peptide isolated on a C-4 VYDAC 10 micron preparative column (22X250 nm) using a 0.1 % trifluoroacetic acid-water/ acetonitrile gradient. The oxidized protein was lyophilized and characterized by mass-spectrometry and amino acid analysis, and shown to be homogeneous. Electro-spray mass spectrometry was used to verify the mass of the synthetic peptides (see Table 1).

Cell lines

Derivatives of the 32D murine myeloid progenitor cell lines were grown in RPMI-1640 medium supplemented with antibiotics, 10% heat-inactivated fetal bovine serum (FBS) and 0.1% medium that was conditioned by the IL-3 producing X63/0 cells. The various sublines expressing ErbB combinations were described previously (Pinkas-Kramarski et al., 1996; Shelly et al., 1998). The human breast cancer cell lines MCF-7, MDA-MB-453 and SKBr-3 were obtained from the American Type Culture Collection (Rockville, MD). MCF-7/ErbB-2, an ErbB-2 overexpressing MCF-7 derivative cell line, has been described (Peles et al., 1993). These human mammary cancer cells were grown in RPMI-1640 medium supplemented with antibiotics and 15% heat-inactivated fetal bovine serum (FBS).

Radiolabeling of ligands and ligand displacement analyses

Recombinant human NDF- β 1177-246 was labeled with IODOGEN (Pierce) as described previously (Karunakaran et al., 1995). For ligand displacement analyses, cell monolayers (10^5 cells per assay) were washed once with binding buffer, and then incubated for 2 hours at 4°C with radiolabeled NDF- β 1 (2 ng/ml) and various concentrations of unlabeled peptides, in a final volume of 0.1 ml. To terminate ligand

binding media were carefully removed and the monolayers washed twice with 1.5 ml binding buffer. Cell-associated radioactivity was determined by using a γ -counter.

Cell proliferation assays

Cells were washed free of IL-3, resuspended in serum-free RPMI-1640 medium at 5×10^5 cells/ml and treated without or with growth factors or IL-3 (1:1000 dilution of medium conditioned by IL-3-producing cells). Cell survival was determined by using the MTT assay as previously described (Pinkas-Kramarski et al., 1996). MTT (0.05 μ g/ml) was incubated for 2 hours at 37°C with the analyzed cells. Living cells can transform the tetrazolium ring into dark blue formazan crystals, that can be quantified by reading the optical density at 540-630 nm after lysis of the cells with acidic isopropanol (Mosman, 1983). For dose-response analyses, serial dilutions of each ligand were added in RPMI-1640 medium, and cells were harvested 24 hours after plating.

Cell growth and cell cycle analyses

MDA-MB-453 cells were seeded at a density of 10^5 per 35-mm dish in RPMI medium containing FBS (10%) and allowed to adhere to the bottom of the plate in the next 12 hours. Then, the medium was replaced with fresh starvation medium containing 0.1% FBS and peptide growth factors as indicated in the legends to figures. Duplicate cultures were used and cell numbers determined at every other day by a dye-exclusion assay (trypan-blue). For cell cycle analyses, MCF-7 cells were plated at a density of 10^5 per a 60-mm culture dish and allowed to adhere overnight. The monolayers were then washed with PBS and incubated with medium containing 0.1% FBS and various peptides, as indicated. Four days later cells were treated with trypsin, washed with PBS, fixed in ice-cold methanol, and stored overnight at -20°C. For staining of DNA the fixed cells were thawed, washed twice with PBS and incubated for 30 min at 37°C with an isotonic solution containing 50 μ g/ml ribonuclease (Boehringer) and propidium iodide (50 μ g/ml, Sigma). The stained cells were analyzed in a fluorescence-activated cell sorter (FACScan; Becton Dickinson). The percentage of cells found at different phases of the cell cycle was determined by using the Cellquest software.

Light and fluorescent microscopy

MCF-7 cells were seeded in 60-mm dishes (10^5 cells/plate) and peptide growth factors added to starvation medium containing 0.1% of FBS. Three to five days later cells were observed using a light microscope (Olympus) and photographed. For immunofluorescence analysis, cells (10^5) were plated on glass coverslips and grown for 24 hours. In the following day the culture medium was replaced by serum-free medium and fourteen hours later cells were treated for 5 minutes at 37°C with peptide growth factors. Subsequent to cell wash with PBS and fixation for 30 minutes in 3 % paraformaldehyde (in PBS), cells were permeabilized for 5 minutes with 0.1% Triton X-100 in PBS. After washing in PBS, cells were incubated for 1 hour with a murine mAb directed to the active form of MAPK. After extensive wash with PBS, the coverslips were incubated with a Cy3-conjugated goat-anti-mouse antibody (from Jackson ImmunoResearch Laboratories, at 1:1000 dilution) and washed twice with PBS. To visualize nuclear DNA, cells were stained with 4,6-diamidino-2-phenylindole (DAPI, dissolved in PBS). Coverslips were viewed with a Zeiss fluorescence microscope in oil immersion.

Lysate preparation for western blot analyses

For receptor activation studies, derivatives of the 32D cell line were resuspended in phosphate-buffered solution (PBS) and incubated at 22°C for 15 minutes before adding growth factors and incubating for five minutes at 37°C. Cells were then pelleted and lysed in ice cold solubilization buffer [50mM Tris-HCl pH 7.5, 150mM NaCl, 1% Noidet-P-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1.5mM EDTA, 1.5mM MgCl₂, 2mM Na-orthovanadate, 1mM phenylmethylsulfonyl fluoride (PMSF), 10µg/ml aprotinin and 10 µg/ml leupeptin] and left on ice for 15 minutes. The whole cell extract was then cleared by centrifugation (12,000 g for 10 min at 4°C), immediately boiled in reducing gel sample buffer, and resolved by 10% SDS PAGE before being transferred onto nitrocellulose. Filters were blocked in TBST buffer (0.02M Tris-HCl [7.5], 0.15M NaCl, 0.05% Tween-20) containing 1% milk for 40

minutes at 22°C, blotted with primary antibodies in TBST overnight at 4°C, followed by conjugation with a secondary antibody linked to horseradish peroxidase and subsequent detection by chemiluminescence (Amersham Corp.). To analyze human mammary cancer cells by western blotting, cells were seeded at 1×10^5 cells per well in 24 well plates in 1 ml of RPM1-1640 medium containing 10% FBS. After 24 hours in culture cells were starved for 12 hours in serum-free medium and then they were treated for 5 min at 37°C with peptide growth factors. After treatment, cells were washed with PBS and gel sample buffer added directly to cell monolayers.

Key Research Accomplishments

The results obtained in the last year can be summarized as follows:

1. A membrane-bound ErbB-2, in the form of a lipid-anchored extracellular domain, can form heterodimers but its ability to negatively regulate signaling appears compromised.
2. Active short synthetic peptides based on a bivalent structure of NDF can be synthesized and they retain specificity to an ErbB-q2/ErbB-3 heterodimer.
3. The length of the linker peptide and modifications of the N-terminal portion of synthetic peptides affect bio-activity of the analogs, and thus may be utilized when designing antagonists.

Reportable Outcomes

The data reported here are currently being prepared for publication. The results obtained will be included in two doctorate theses. We have not reported the data in meetings; when completed the work will be presented in an abstract form along with submission as a manuscript.

Conclusions (discussion)

In this report we describe the synthesis and biological activities of short peptide analogs of NDF. All tested activities of NDF were retained by the active analogs, albeit with reduced potency. These include specific binding to the cell surface (Fig. 8), activation of both tyrosine phosphorylation (Fig. 4) and MAPK (Figs. 4, 6 and 7), and stimulation of cell proliferation (Figs. 2, 3, 5, and 7). These results suggest that the peptide analogs may act as partial agonists of the NDF pathway. Comparison of several peptides

implied that in addition to intactness of the two termini of the EGF-like domain, a linker whose length is 19 Å or longer is sufficient for activity restoration (Table 1). This rather limited structural constraint is surprising given the wealth of available information on the multiple contacts EGF-like ligands and ErbB receptor can form at their interface. For example, not only residues of the NH₂ terminus of NDF, but also portions of the two β-turns, the major β sheet and the COOH-terminal region are essential for binding to ErbB-3 and ErbB-4 (Jones et al., 1998). Likewise, the receptor interfaces of EGF and TGFα might encompass one complete side of the molecule with several strong binding determinants [reviewed in (Groenen et al., 1994)]. Thus, the ability of the short peptide analogs to stimulate cells via ErbB proteins must be mediated by only a fraction of the necessary interface. This may explain why the binding affinity of the peptides is severely reduced (Fig. 8). Moreover, the short peptides have completely lost the ability to recognize ErbB-4 (Fig. 2), and their residual interaction with the other direct receptor of NDF, namely ErbB-3, strictly depends on the presence of ErbB-2. Unlike the combination of ErbB-2 with ErbB-3, co-expression of ErbB-2 with ErbB-4 enabled no response to the short peptides, indicating a difference between the two NDF receptors. Although the two receptors use similar determinants in their interactions with NDF/neuregulin (Ballinger et al., 1998), alanine-scanning mutagenesis suggests that ErbB-3 has more stringent requirements for binding than ErbB-4 (Jones et al., 1998). Differences in ligand binding have been previously implied by the primary structures of the two receptors (Plowman et al., 1993), their binding affinities (Tzahar et al., 1994), antibody recognition (Chen et al., 1996) and differential binding of NDF mutants (Jones et al., 1998).

It is important to note that previous attempts to minimize other ErbB ligands have essentially failed. Thus, a cyclic fragment representing loop-B of the rodent EGF retained only 0.01% of the activity of the intact ligand (Komoriya et al., 1984), but a similar peptide derived from the human ligand has completely lost activity (Han et al., 1988). Many other fragments of EGF and TGFα have no biological activity and they do not bind to the EGF-receptor [reviewed in (Groenen et al., 1994)]. An exception is a derivative of the C-loop of TGFα which can displace EGF binding but acts as an

antagonist rather than as an agonist (Nestor et al., 1985). However, it is unclear whether or not this peptide binds to ErbB-1 or it indirectly inhibits binding of EGF through another cell surface protein (Eppstein et al., 1989). Thus the observed activity of the short NDF analogs we described may reflect some unusual properties of their unique receptor, namely: the ErbB-2/ErbB-3 heterodimer. Out of the 10 possible homo- and hetero-dimeric receptor combinations, the short analogs can recognize and activate only this receptor pair (Figs. 1 and 2, and data not shown). Indeed, the binding properties of the ErbB-2/ErbB-3 heterodimer are unusual: Although neither ErbB-2 nor ErbB-3 can bind EGF when each receptor is singly expressed, their co-expression can reconstitute a low affinity binding site for not only EGF but also for betacellulin (Alimandi et al., 1997; Pinkas-Kramarski et al., 1998) and epiregulin (Shelly et al., 1998). This extended ligand specificity is limited because the other ErbB-1 ligands, namely: TGF α , the heparin-binding EGF and amphiregulin do not recognize the heterodimer. Comparison of EGF and TGF α revealed the involvement of the B-loop of EGF in heterodimer recognition (Pinkas-Kramarski et al., 1998). This is consistent with the ability of biregulin, a ligand containing the N-terminus of NDF and the A-, B- and C-loops of EGF (Barbacci et al., 1995), to specifically activate the ErbB-2/ErbB-3 heterodimer, in addition to its ability to recognize all ErbB-1-containing combinations (Barbacci et al., 1995; Tzahar et al., 1997). Another reflection of the unusual binding properties of the ErbB-2/ErbB-3 combination is its function as the exclusive receptor of a poxvirus-encoded ligand, the Myxoma growth factor [MGF, (Tzahar et al., 1998)].

It is interesting to address the mechanism underlying the ability of the synthetic analogs to recognize the ErbB-2/ErbB-3 heterodimer. Because ErbB-3 cannot recognize the synthetic ligands when it is singly expressed (data not shown), which is also true for EGF and biregulin binding (Alimandi et al., 1997; Pinkas-Kramarski et al., 1998; Tzahar et al., 1997), ErbB-2 must be involved in ligand recognition. While an indirect effect cannot be excluded, several lines of evidence imply the existence of direct interactions between ErbB-2 and ligands presented to it by ErbB-3. Thus, a relatively large group of monoclonal antibodies to ErbB-2 can accelerate dissociation of NDF (Klapper et al., 1997), as well as EGF (Pinkas-Kramarski et al., 1998), from the ErbB-2/ErbB-3

heterodimer. These observations raise the possibility that ErbB-2 is endowed with a ligand binding cleft. Indeed, sedimentation equilibrium analysis (Horan et al., 1995) and measurements using surface plasmon resonance (Tzahar et al., 1997) detected very low binding affinity of ErbB-2 to NDF. Structural analyses of the heterodimer by using truncated versions of ErbB-3 implied that this rather low affinity is significant in the context of a membrane-anchored receptor complex (Tzahar et al., 1997). Direct ligand binding to ErbB-2 is consistent with the ability of the overexpressed protein to decelerate the rate of NDF dissociation (Karunakaran et al., 1996). This model may also explain how NDF can displace EGF in an ErbB-2 dependent manner (Karunakaran et al., 1995). Thus, a plausible scenario attributes the recognition of the heterodimer to simultaneous and cooperative interactions of the short NDF analogs with both ErbB-3 and ErbB-2. On the basis of previous reports (Barbacci et al., 1995; Jones et al., 1998) and the bivalence model of NDF (Tzahar et al., 1994; Tzahar and Yarden, 1998), we assume that the shared N-terminus of the analogs interacts with ErbB-3, whereas the C-terminus recognizes ErbB-2.

One interesting implication of our observation is the functional role of the core of NDF. Because the two termini of the EGF-like domain are sufficient for recognition of the ErbB-2/ErbB-3 heterodimer, it is conceivable that a major role of the core is to maintain the appropriate orientation and distance between the two receptor binding sites. This possibility is supported by the three-dimensional structure of NDF (Jacobsen et al., 1996): the two sites are remote from each other in the folded protein. Moreover, the N-terminus of NDF is significantly flexible (Fairbrother et al., 1998). In addition, the intervening amino acid sequence, through well-defined disulfide and hydrogen bonding, is maintained in an extremely compact structure. Perhaps the most important single residue is the highly conserved Arginine-44, which is absolutely required for binding of EGF to ErbB-1 (Engler et al., 1992) and significantly affects binding of NDF to ErbB-3 and ErbB-4 (Jones et al., 1998). This residue forms three hydrogen bonds with the N-terminal half of NDF and therefore its involvement may be purely structural. Because loop-B of EGF and TGF α , more than the corresponding region of NDF, is involved in receptor binding (Pinkas-Kramarski et al., 1998; Richter et al., 1995), linking of the

termini of ErbB-1 ligands (e.g., EGF and TGF α) may not restore receptor recognition. Despite being limited to NDF and the ErbB-2/ErbB-3 heterodimer, our observations may open the way for mutational analyses of the two putative binding sites of NDF. Furthermore, grafting sequences derived from different ErbB ligands into the C-terminus of analogous peptides may provide a convenient approach to study the elusive binding specificity of ErbB-2. Such analyses will aid the design of an antagonist of the ErbB-2/ErbB-3 heterodimer. This heterodimer is not only the most stable NDF-induced receptor combination (Tzahar et al., 1996): its mitogenic activity exceeds that of other ErbB complexes (Pinkas-Kramarski et al., 1996) and it is the most abundant receptor species in some human carcinoma cells (Chen et al., 1996). Consistent with these properties, co-expression of ErbB-2 with ErbB-3 can confer a transformed phenotype (Alimandi et al., 1995; Wallasch et al., 1995). Thus, the synthetic analogs we examined are expected to allow highly specific targeting of toxin- or drug-conjugated ligands to this cancer-promoting receptor complex.

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Appendices

Table 1 Amino-acid sequences of NDF and derived analogs.

The amino (N) terminal region of NDF- β 1 (residues 177-181) and the C-terminal portion (F, residues 222-226) are shown in bold type. The intervening sequences in NF are a Ser-Gly-Ser triad flanked at each side by an amino caproic acid (Aca) moiety. Note that the N-termini of peptides designated FN, Rev-FN and Ac-NF are blocked by an acetyl (Ac) group. The indicated molecular weights were determined by mass spectrometry and the lengths calculated on the basis of structure prediction. ND-compounds currently being analyzed.

| Name | Sequence | Length Å | Mr | Cell Growth (D23 cells) |
|-------------|---|---------------|-------------|----------------------------------|
| NDF β | SHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCPNEFTGDRCQNYVM | 15.9/16. 6 | 5740 | + |
| NF' | NH2- SHLVK -Aca----G----Aca- QNYVM -CONH2 | 19.2 | 1500 | + |
| NF | NH2- SHLVK -Aca- S-G-S-Aca- QNYVM -CONH2 | 26.5 | 1675 | + |
| Ac-NF | [Ac]- SHLVK -----S-G-S----- QNYVM - [NH2] | 9.6 | 1490.5 8 | - |
| FN | [Ac]- QNYVM -Aca-S-G-S-Aca- SHLVK - [NH2] | 26.5 | 1716.9 4 | - |
| Rev-FN | [Ac]- MVYHQ -Aca-S-G-S-Aca- KVLNS -[NH2] | 26.5 | 1716.9 4 | - |
| | [H]- SHLVKSGSQNYVM -[NH2] | 9.6 | 1448.5 8 | ND |
| | [H]- SHLVK -Aca-SCS-Aca- QNYVM -[NH2] | 26.5 | 1720.6 7 | ND |
| | [H]- NSDSE -Aca-SGS-Aca- QNYVM -[NH2] | 26.5 | 1642.3 4 | ND |

Legends to Figures

Figure 1 Proposed structures of NDF analogs. A model structure of the EGF-like domain of NDF was predicted by using the average NMR structure (entry 1HRE, Protein Data Bank). Residues 1-5 (corresponding to residues 177-181 of the full-length molecule) are shown in blue, and residues 46-50 (222-226 in full-length NDF) are in red. The intervening sequence is shown as a ribbon diagram (yellow). The backbones of the chemical linkers of NF (green) and NF' (light green) are shown in their most extended form.

Figure 2 Proliferative responses of ErbB-expressing derivatives of 32D myeloid cells to NDF and short synthetic analogs.

The following sublines of 32D cells were tested for cell proliferation by performing the MTT assay in the presence of the indicated peptides. D2 (closed circles), D3 (closed squares) and D23 (open squares) cells expressing respectively ErbB-2, ErbB-3, and a combination of ErbB-2 and ErbB-3. For control we used the parental 32D cells (open circles). Cells were deprived of IL-3 and plated in media containing serial dilutions of each peptide. NF is a peptide comprised of the N- and C-termini of NDF, FN is a similar peptide where the termini were exchanged, and Ac-NF is an N-terminally acetylated short derivative of NF (see Table 1). The MTT cell proliferation assay was performed 24 hours later. Results are presented as fold induction over the untreated control cells, and are the mean \pm S.D. of four determinations. The experiment was repeated at least three times.

Figure 3 Survival of ErbB-expressing 32D cells in the presence of NDF analogs.

The indicated sublines of 32D cells were incubated for various time intervals in presence (closed triangles) or absence of IL-3 (open squares), or with one of the following peptides (each at 100 ng/ml): NDF (open circles), NF (closed circles) and NF' (closed squares). Note that NF' is a derivatives of NF whose stuffer linking the N- and C-terminal portions of NDF is shorter than the linker of NF (see Table 1). D4 and D24 cells respectively express ErbB-4 alone or in combination with ErbB-2.

Cell survival was determined with the indicated cell lines, essentially as described in A. In addition to NDF (open circles), IL-3 (closed triangles) and control cultures with no added factor (open squares) we tested the following control peptides: rev-FN (closed squares), FN (diamonds), and Ac-NF (closed circles). The MTT assay was performed daily and the reported data are the means \pm S.D. of four determinations. Each experiment was repeated three times.

Figure 4 Receptor phosphorylation and MAPK activation induced by NDF and related short peptides.

D23 cells (10^6 cells per lane) were incubated for 5 min at 37° C with the indicated peptides (at 50 ng/ml). At the end of the incubation time, whole cell lysates were prepared, resolved by gel electrophoresis, and subjected to immunoblotting with either an antibody to phosphotyrosine (P-TYR), an antibody specific to the active doubly-phosphorylated form of the MAPK (P-MAPK), or with an antibody recognizing the unmodified form of Erk/MAPK.

Figure 5 Growth curves of MDA-MB-453 epithelial cells treated with either NDF or derivative peptides.

Panel A- MDA-MB453 human breast cancer cells (10^5) were plated in medium containing 0.1% bovine serum in the presence of either NDF (open circles), NF (closed circles), or NF' (closed squares), each at 50 ng/ml. Control cultures were incubated in medium (0.1% serum) with no added factors (open squares). The cells were counted each other day.

Panel B-Cell survival was determined as in A except that the following control peptides were tested: rev-FN (closed squares), FN (open diamonds) and Ac-NF (closed circles). Shown are the results of one of three experiments that were independently performed. The average and the standard deviations (bars) represent the variations displayed by three separate plates.

Figure 6 MAPK activation by NDF analogs in epithelial cells.

MDA-MB453 were incubated for the indicated time intervals with NDF, NF, or NF' (each at 50 ng/ml). At the end of the incubation interval (shown in minutes), whole cell lysates were prepared and cleared from debris and nuclei. Lysates were subjected to immunoblot analysis with an antibody to the active doubly phosphorylated form of the MAPK (P-MAPK), or with an antibody specific to the native unmodified form of Erk/MAPK.

Immunofluorescence analysis of MDA-MB453 cells was performed with an antibody specific to the active doubly phosphorylated form of Erk/MAPK. Following 14 hours of starvation, 10^5 MDA-MB453 cells were incubated for 5 min at 37°C with the indicated ligands (each at 50 ng/ml). The cells were then fixed, permeabilized and stained with either DAPI (blue) or an antibody specific to the active form of the MAPK (red). Note nuclear localization of the active form of the kinase.

Figure 7 Effects of NDF analogs on survival of MCF-7 breast cancer epithelial cells.

Panel A-MCF-7 cells (10^5 cells) were plated in 35-mm dishes in medium containing 0.1% bovine serum. The medium was supplemented with either NDF or the indicated peptide analogs. Control cells were plated in medium (0.1 % serum) with no added factor (*Control*). Shown are photographs representative of six independent plates. Cells were visualized three days after addition of the peptides as detailed under Material and Methods.

Panel B-MCF-7 cells were stimulated for 5 minutes at 37°C with the indicated factors (each at 50 ng/ml) and their whole cell lysates subjected to immunoblotting with antibodies specific to the active form of the MAPK.

Panel C-Cell cycle analysis was performed by using a cell sorter four days after adding the indicated peptides (50 ng/ml) to MCF-7 cells. The fraction of cells found at the sub-G1 phase of the cell cycle after the indicated treatments is shown.

Panel D-Representative cell cycle profiles of MCF-7 cultures treated for four days with medium containing 0.1% bovine serum in the absence or presence of the indicated peptides (at 50 ng/ml).

Figure 8 Displacement analyses of cell-bound NDF with peptide analogs.

Displacement analyses of radiolabeled ^{125}I -NDF were performed with SKBr-3 cells. Cells (2×10^5 per assay) were incubated for 2 hours at 4°C with the radiolabeled ligand (2 ng/ml) in the absence or presence of increasing concentrations of an unlabeled NDF (open squares), NF (open circles), NF' (open triangles), Ac-NF (closed circles) and EGF (closed squares). Non-specific binding of NDF was determined in the presence of 100-fold excess of the unlabeled ligand. Each data point represents the mean (less than 10% variation) of two determinations performed in three independent experiments.

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List of Personnel

Two Ph.D. students, Ms. Maya Shelly and Mr. Shlomo Oved were paid by the IDEA grant.

Figure 1



Figure 2

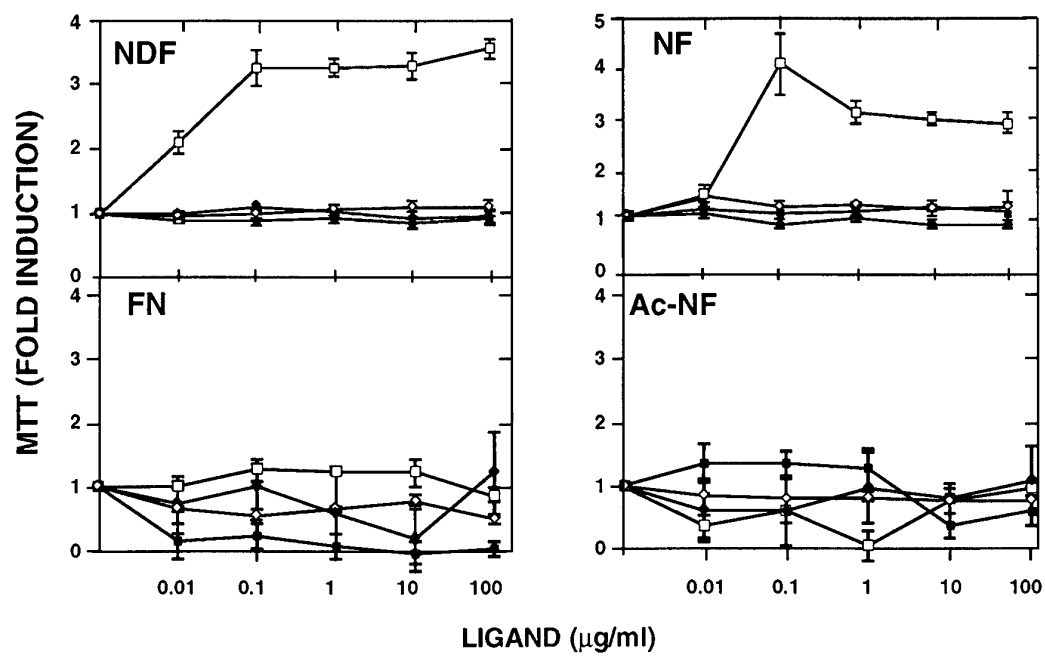


Figure 3

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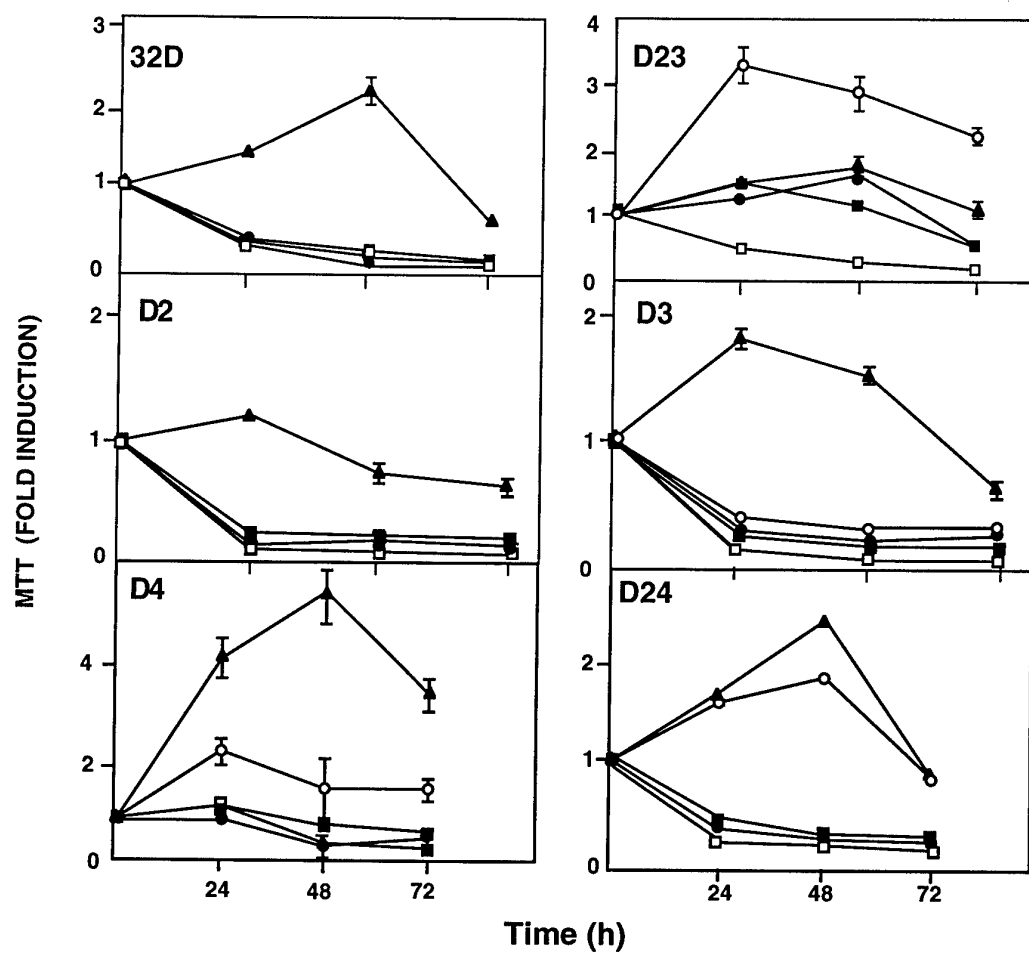


Figure 4

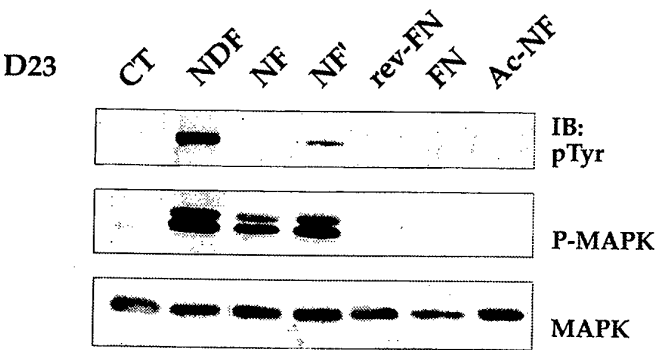
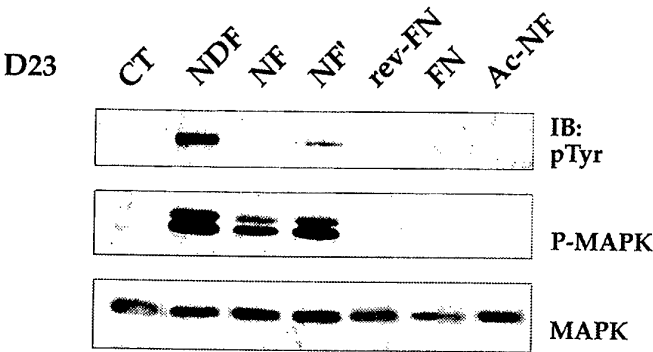


Figure 5

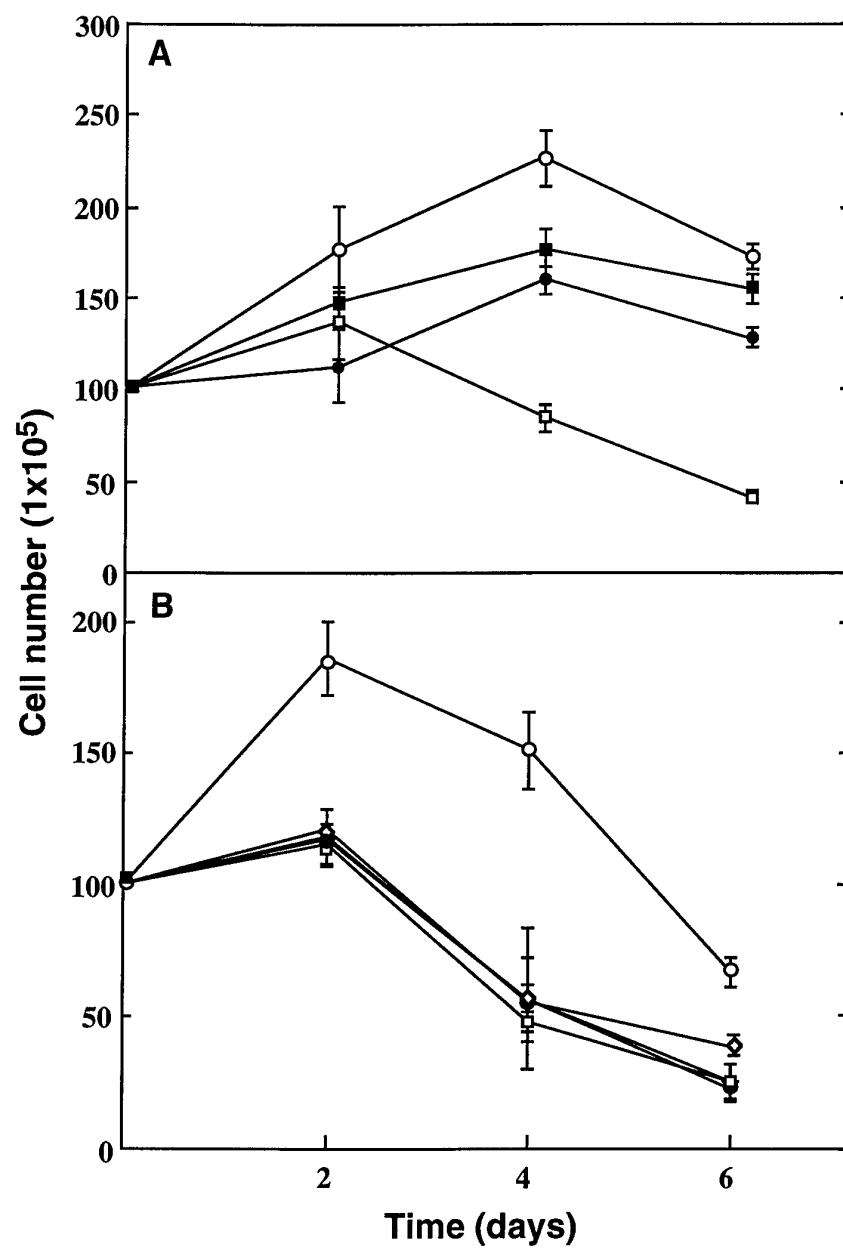


Figure 6

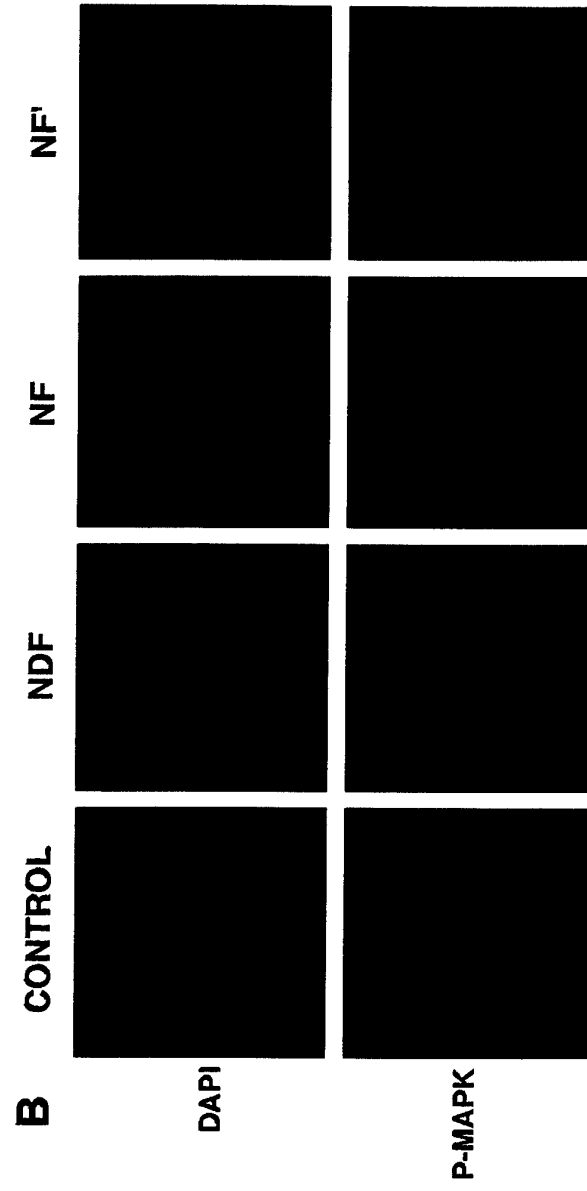


Figure 6

A

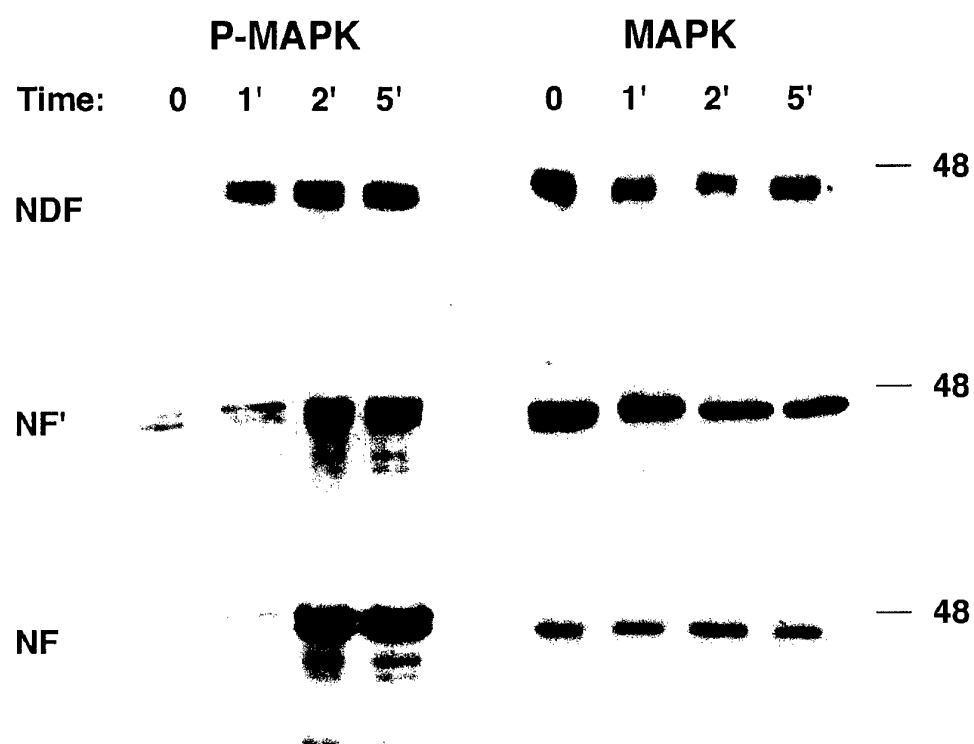
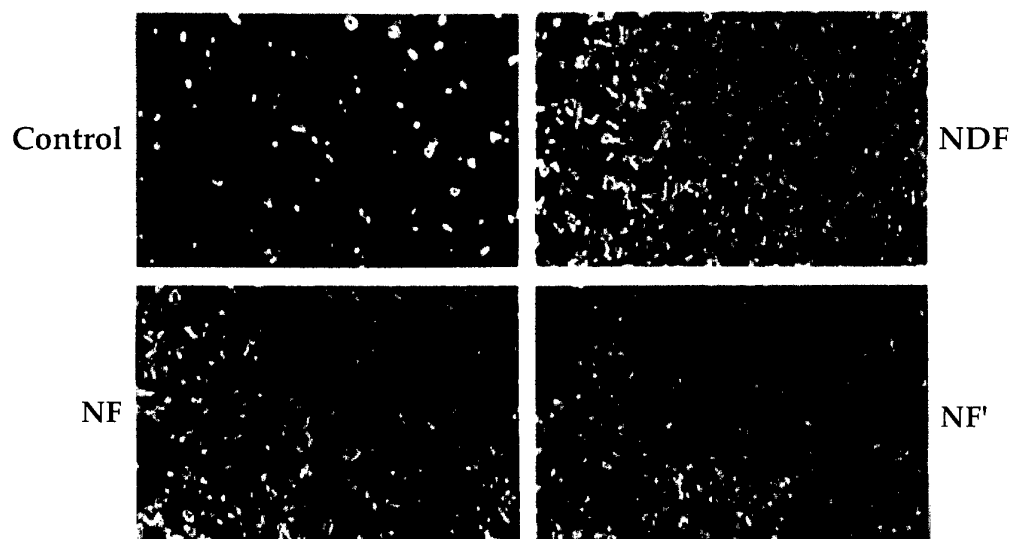


Figure 7

A



B

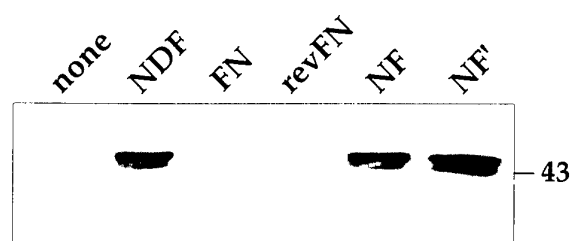
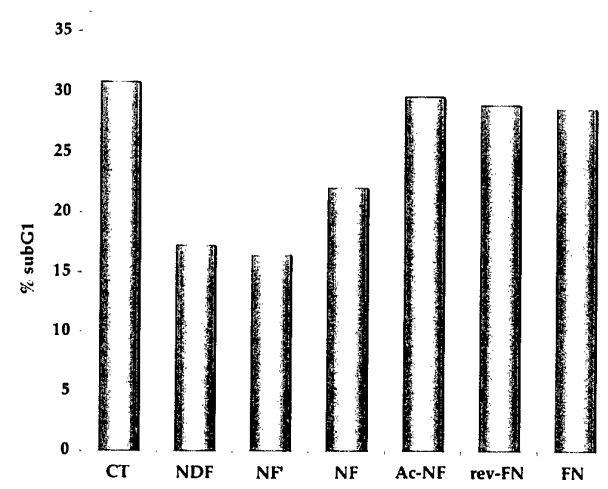


Figure 7

C



D

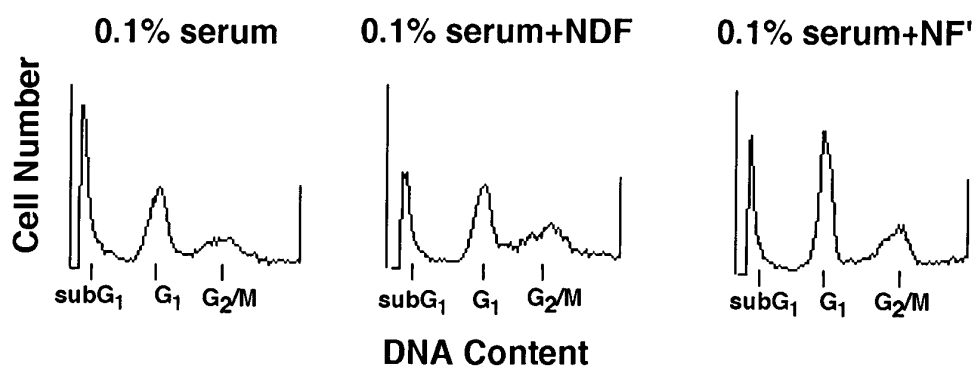
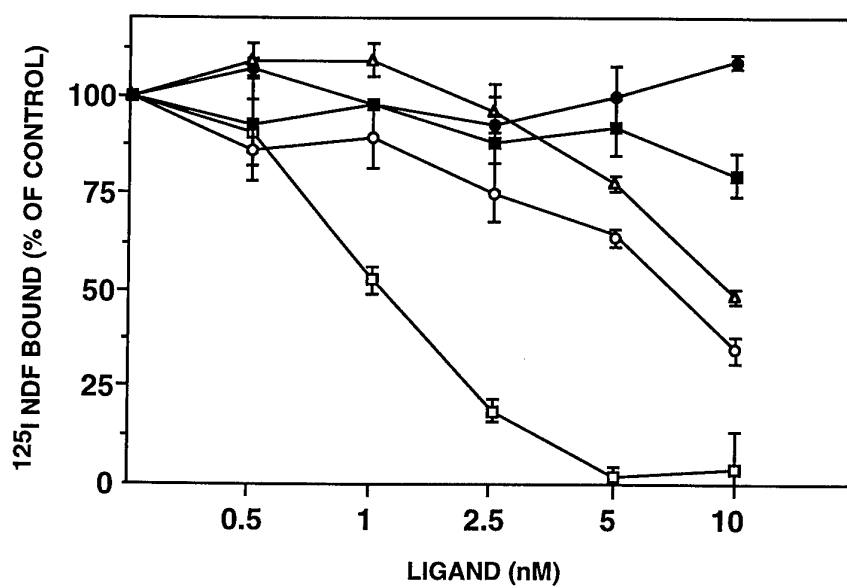


Figure 8



ErbB Tyrosine Kinases and the Two Neuregulin Families Constitute a Ligand-Receptor Network

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The recently isolated second family of neuregulins, NRG2, shares its primary receptors, ErbB-3 and ErbB-4, and induction of mammary cell differentiation with NRG1 isoforms, suggesting functional redundancy of the two growth factor families. To address this possibility, we analyzed receptor specificity of NRGs by using an engineered cellular system. The activity of isoform-specific but partly overlapping patterns of specificities that collectively activate all eight ligand-stimulatable ErbB dimers was revealed. Specifically, NRG2- β , like NRG1- α , emerges as a narrow-specificity ligand, whereas NRG2- α is a pan-ErbB ligand that binds with different affinities to all receptor combinations, including those containing ErbB-1, but excluding homodimers of ErbB-2. The latter protein, however, displayed cooperativity with the direct NRG receptors. Apparently, signaling by all NRGs is funneled through the mitogen-activated protein kinase (MAPK). However, the duration and potency of MAPK activation depend on the identity of the stimulatory ligand-receptor ternary complex. We conclude that the NRG-ErbB network represents a complex and nonredundant machinery developed for fine-tuning of signal transduction.

One of the relatively simple systems of signal transduction by a polypeptide growth factor is the mechanism controlling vulva formation in the nematode *Caenorhabditis elegans* (reviewed in reference 33). The most ancient epidermal growth factor (EGF)-like ligand, Lin-3, which is expressed by the anchor cell, binds to the Let-23 transmembrane tyrosine kinase on the surface of the closely apposed vulva precursor cell. The latter is then directed to a vulval fate through a biochemical cascade that sequentially activates a small GTP binding protein and a series of protein kinases, culminating in the mitogen-activated protein kinase (MAPK). A remarkably expanded version of this signaling module exists in mammals (reviewed in reference 6). Four receptors, whose structures are homologous to Let-23, and a few dozen ligands, all sharing the three-loop structure of EGF, form an interactive system with a large potential for signal diversification. In addition to the multiplicity of components, the modern version of the module is characterized by diversity: one ErbB protein, ErbB-3, is devoid of tyrosine kinase activity (25), and another, ErbB-2, binds no known EGF-like factor with high affinity (28, 61). Likewise, the various ligands carry, in addition to the EGF-like motif, a variety of structural domains thought to allow interaction with extracellular components. For example, the heparin binding EGF-like factor includes a heparan sulfate binding moiety (26), and the Neu differentiation factor (NDF, also called neuregulin 1 [NRG1], or heregulin) carries an immunoglobulin (Ig) domain (27, 37, 63).

A combination of in vitro experiments and gene targeting in mice implies that the mammalian ErbB module, like its inver-

tebrate counterparts in worms and in flies (46), is involved with fate determination of several cell lineages. Thus, ErbB-1, and some of its ligands, control the development of specific types of epithelia (42), whereas NRG1 and its receptor, ErbB-4, play an essential role in formation of trabeculae in the embryonic heart (21, 41). Other functions of neuregulins include strengthening of the neuromuscular synapse (19); differentiation of myelin-producing cells, both Schwann cells (17) and oligodendrocytes (8); and lobulo-alveolar differentiation in the mammary gland (65). Each of these physiological roles depends on a specific combination of receptors, which likely represents the necessity for receptor heterodimerization, as opposed to homodimerization, for signaling. The importance of receptor heterodimerization, a process that does not exist in the invertebrate forms of the module, is exemplified by gene targeting of *erbB-2*: Despite the fact that this receptor has no direct ligand, the resulting phenotype is almost identical to those of *NRG1*- and *erbB-4*-targeted mice (35).

Through functional inactivation of ErbB-2 in cultured cells (4, 23, 24, 30) and ectopic expression of single or specific pairs of ErbB proteins in defined cellular contexts (11, 15, 49, 52, 62, 67), it became clear that the mammalian ErbB module functions as a signaling network. In general, homodimers of ErbBs are either devoid of biological activity (i.e., ErbB-3 homodimers) or are weakly active (e.g., ErbB-1 homodimers), and heterodimeric combinations are strongly active. Most potent are ErbB-2-containing combinations, whose signaling is prolonged because of an ErbB-2-mediated deceleration of ligand dissociation (30). Importantly, each ligand appears to be characterized by a distinct ability to stabilize specific homo- and heterodimeric receptors (48), thus enhancing the diversification potential of the network. According to a recently proposed model, ligand-specific dimerization is due to bivalence of EGF-like growth factors: their high-affinity site binds a pri-

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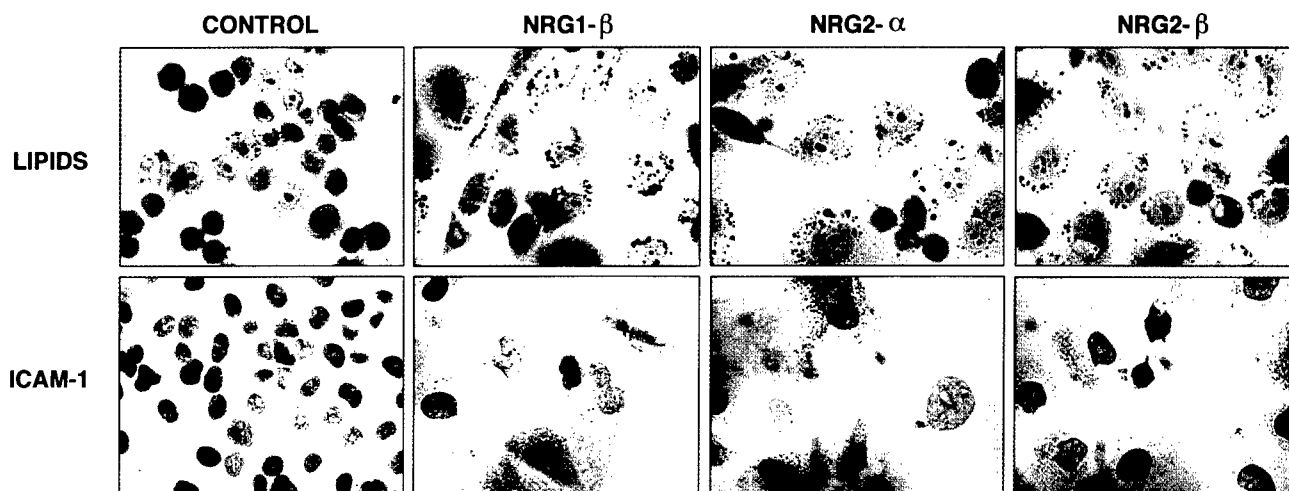


FIG. 1. Induction of cellular differentiation by neuregulin isoforms. AU-565 human mammary cancer cells, which express all four ErbB proteins, were plated in chamber slides and then incubated for 4 days in the absence (CONTROL) or presence of the indicated NRG isoforms (each at 50 ng/ml). Cells were stained with either Oil red O, to visualize neutral lipids, or with an antibody to ICAM-1. Antibody visualization was performed by using a biotinylated rabbit anti-mouse IgG, followed by an alkaline phosphatase-conjugated streptavidin and a red chromogen. Note the appearance of lipid droplets (yellow) and ICAM-1 (red stain) in NRG-treated cells. The magnification used was $\times 444$ (lipid staining) or $\times 296$ (ICAM-1 staining).

mary receptor (ErbB-1, -3, or -4), and a low-affinity site whose specificity is broad selects the interacting receptor with some preference for ErbB-2 (61).

On the basis of the lines of evidence described above, it seems safe to conclude that multiplicity of receptors and ligands increases the functional versatility of the mammalian ErbB signaling module. Therefore, the recent isolation of an additional family of EGF-like ligands of ErbB proteins, denoted NRG2 (7, 9, 12), is expected to further enhance signal diversification. However, receptor specificity of NRG2s appears to be shared with that of NRG1s (7, 9, 12). This observation implies an overlap of signaling pathways by the two NRG families and possible functional redundancy. We aimed at this possibility by making use of synthetic and recombinant forms of NRG2 and NRG1 (α and β isoforms of each), respectively, and a series of interleukin 3 (IL-3)-dependent cell lines expressing defined combinations of ErbB proteins. Our results reveal significant differences between the two isoforms of NRG2. Moreover, each of the four NRG isoforms is distinct in terms of its ErbB specificity. For example, NRG2- α emerges as the broadest specificity factor, whereas the ranges of specificities of NRG2- β and NRG1- α are relatively narrow. Taken together, these results support the notion that the multiple ErbB ligands, through differences in affinity and in specificity to certain receptor dimers, expand the diversification potential of the ErbB signaling module.

MATERIALS AND METHODS

Materials and antibodies. EGF was purchased from Sigma (St. Louis, Mo.), and recombinant NDF- α and NDF- β preparations (EGF-like domains) were from Amgen (Thousand Oaks, Calif.). Radioactive materials were from Amersham (Buckinghamshire, United Kingdom). Iodogen and bis(sulfosuccinimidyl) suberate (BS³) were from Pierce. Monoclonal antibodies to ErbB proteins (14, 32) were used for immunoprecipitation. A monoclonal antiphosphotyrosine antibody (PY-20; Santa Cruz Biotechnology) was used for Western blot analysis. A murine monoclonal antibody to an active form of MAPK (doubly phosphorylated on both threonine and tyrosine residues of the TEY motif) has been described previously (66). The composition of the buffered solutions has been described previously (62).

Peptide synthesis. NRG2 isoforms were synthesized on an Applied Biosystems (ABI) 430A peptide synthesizer with standard *tert*-butyloxycarbonyl (*t*-Boc) chemistry protocols as provided (version 1.40; *N*-methylpyrrolidone-hydroxybenzotriazole). Only the EGF-like domains of NRG2- α and NRG2- β (7, 9, 12)

were synthesized. Acetic anhydride capping was employed after each activated ester coupling. The peptides were assembled on phenylacetamidomethyl polystyrene resin by using standard side chain protection, except for the use of *t*-Boc-Glu(*O*-cyclohexyl) and *t*-Boc-Asp(*O*-cyclohexyl). The peptides were deprotected by using the low-high hydrofluoric acid (HF) method (59). In each case, the crude HF product was purified by reverse-phase high-performance liquid chromatography (HPLC) (C₁₈ Vydac; 22 by 250 mm), diluted without drying in folding buffer (1 M urea, 100 mM Tris [pH 8.0], 1.5 mM oxidized glutathione, 0.75 mM reduced glutathione, 10 mM methionine), and stirred for 48 h at 4°C. Folded, fully oxidized peptides were purified from the folding mixture by reverse-phase HPLC and characterized by electrospray mass spectroscopy. Peptide quantities were determined by amino acid analysis. Disulfide bonding was analyzed in the following manner. First, the peptide was cleaved with cyanogen bromide (CNBr), which opened up the peptide for further digestion. After removal of CNBr, the peptide was sequentially digested with proteolytic enzymes in order to obtain cleavage between the cysteines. Samples were analyzed by capillary liquid chromatography coupled with electrospray ionization mass spectrometry. The disulfide bonding pattern was determined by using the molecular weights of the fragmented peptides and was shown to be the expected C-1-C-3, C-2-C-4, and C-5-C-6.

Cell lines. The establishment of a series of IL-3-dependent 32D myeloid cells expressing all combinations of ErbB-1, ErbB-2, and ErbB-3 has been described previously (49). To generate an ErbB-4-expressing derivative of 32D cells, we used a long terminal repeat (LTR)-erbB-4 expression vector that was electroporated into 32D cells as described previously (47). Cell lines coexpressing ErbB-2 or ErbB-3, together with ErbB-4, were established by transfection of the pLXSHD retroviral vector (57) directing ErbB-4 expression into ErbB-2- or ErbB-3-expressing cells (D2 and D3 cell lines, respectively) by electroporation (BioRad GenePulser set at 400 V and 250 μ F). After a 24-h-long recovery, cells were selected for 4 to 5 weeks in medium containing histidinol (0.4 mg/ml; Boehringer). Clones expressing the two receptors were identified by Western blotting and isolated by limiting dilution. Due to differences in promoter potency, the selected cell line that singly expresses ErbB-4 (D4 cells) contained approximately 10- to 12-fold more ErbB-4 than cell lines expressing the combinations of ErbB-4 with ErbB-2 (D24 cells) or with ErbB-3 (D34 cells).

Radiolabeling of ligands, covalent cross-linking, and ligand binding analyses. Growth factors were labeled with Iodogen (Pierce) as described previously (31). The range of specific activity varied between 2×10^5 cpm/ng (NRG2- α) and 3×10^5 cpm/ng (NRG1- β and NRG2- β). For covalent cross-linking analysis, cells (10⁷) were incubated on ice for 1.5 h with either ¹²⁵I-NRG2- α or ¹²⁵I-NRG2- β (each at 250 ng/ml). The chemical cross-linking reagent BS³ was then added (1 mM), and after 1.5 h on ice, cells were pelleted and solubilized in solubilization buffer. For ligand displacement analyses, 10⁶ cells were washed once with binding buffer and then incubated for 2 h at 4°C with radiolabeled NRG1- β (5 ng/ml) and various concentrations of an unlabeled ligand, as indicated, in a final volume of 0.2 ml. Nonspecific binding was determined in the presence of a 100-fold molar excess of the unlabeled ligand. To terminate ligand binding, each reaction tube was washed once with 0.5 ml of binding buffer and loaded on top of a 0.7-ml cushion of bovine serum. The tubes were spun (12,000 \times g, 2 min) in order to remove the unbound ligand.

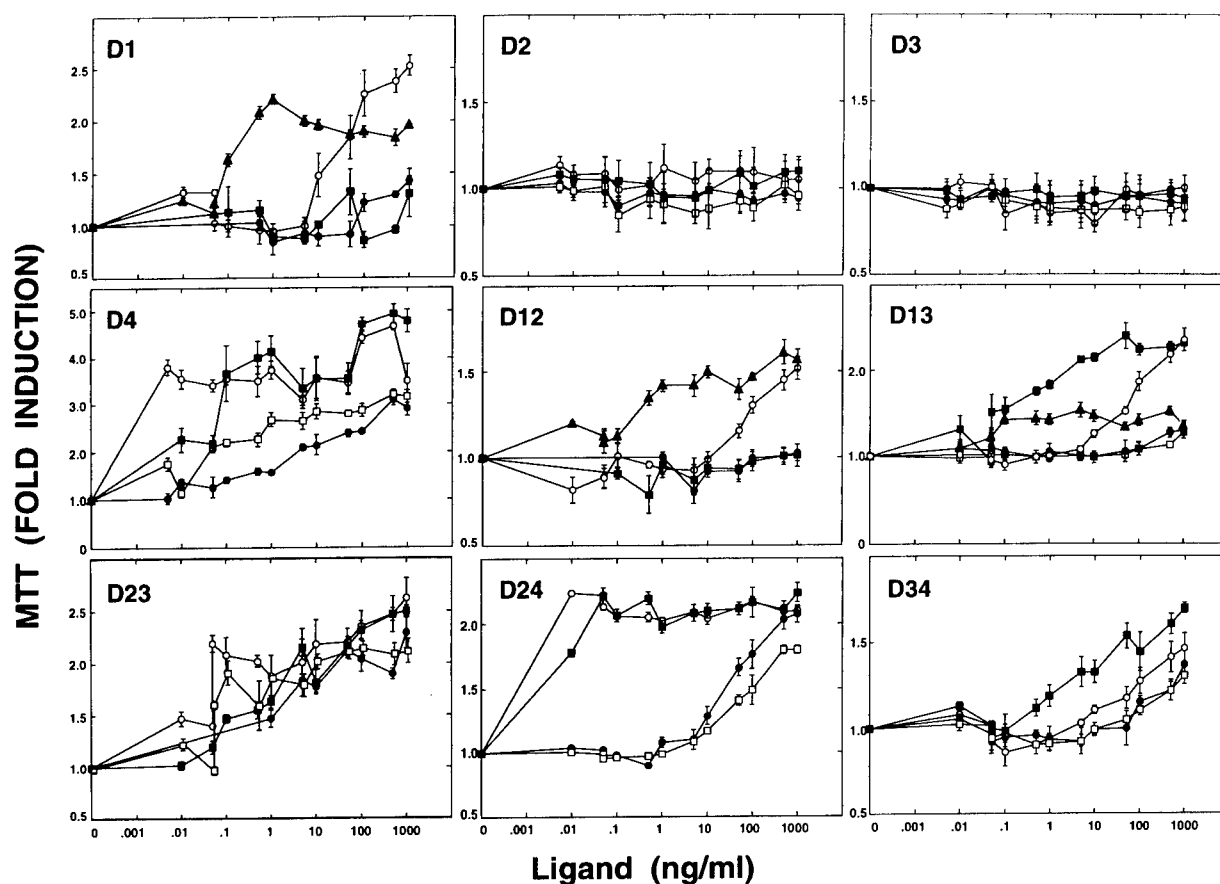


FIG. 2. Proliferative responses of ErbB-expressing derivatives of 32D cells to the four major NRG isoforms. The indicated sublines of 32D cells were tested for cell proliferation by using the MTT assay. Cells were deprived of serum factors and IL-3 and then plated at a density of 5×10^5 cells/ml in media containing serial dilutions of EGF (closed triangles), NRG1- α (open squares), NRG1- β (solid squares), NRG2- α (open circles), or NRG2- β (solid circles). The MTT assay was performed 24 h later. Results are presented as fold induction over the control untreated cells and are the mean \pm standard deviation of four determinations. Each experiment was repeated at least twice. Note that no responses were observed with cells expressing either ErbB-2 or ErbB-3 alone, but these cell derivatives retained a response to IL-3.

Lysate preparation, immunoprecipitation, and Western blotting. For analysis of total cell lysates, gel sample buffer was added directly to cell monolayers or suspensions. For other experiments, solubilization buffer was added to cells on ice. Cells were scraped with a rubber policeman into 1 ml of buffer, transferred to microtubes, mixed harshly, and centrifuged ($10,000 \times g$, 10 min at 4°C). Rabbit antibodies were directly coupled to protein A-Sepharose beads while shaking for 20 min. Mouse antibodies were first coupled to rabbit anti-mouse IgG and then to protein A-Sepharose beads. The proteins in the lysate supernatants were immunoprecipitated with aliquots of the protein A-Sepharose-antibody complex for 1 h at 4°C . Immunoprecipitates were then washed three times with 20 mM HEPES buffered at pH 7.5–150 mM NaCl–0.1% Triton X-100–10% glycerol (HNTG; 1 ml each wash) prior to being heated (5 min at 95°C) in gel sample buffer. Samples were resolved by gel electrophoresis through 7.5% acrylamide gels and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 2 h in TBST buffer (0.02 Tris-HCl buffered at pH 7.5, 0.15 M NaCl, and 0.05% Tween 20) containing 1% milk and blotted with 1 μg of primary antibodies per ml for 2 h, followed by blotting with 0.5 μg of secondary antibody per ml linked to horseradish peroxidase. Immunoreactive bands were detected with an enhanced chemiluminescence reagent (Amersham Corp.).

Cell proliferation assays. Cells were washed free of IL-3, resuspended in RPMI 1640 medium at 5×10^5 cells/ml, and treated without or with growth factors (at 100 ng/ml, unless otherwise indicated) or IL-3 (1:1,000 dilution of conditioned medium). Cell survival was determined by using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl] tetrazolium bromide (MTT) assay as previously described (49). MTT (0.05 mg/ml) was incubated with the cells analyzed for 2 h at 37°C . Living cells can transform the tetrazolium ring into dark-blue formazan crystals that can be quantified by reading the optical density at 540 to 630 nm after lysis of the cells with acidic isopropanol (43).

Cellular differentiation assays. AU-565 human mammary cancer cells were plated in chamber slides (Lab-Tek) and then incubated for 4 days in the absence or presence of ligands (50 ng/ml). Cells were stained with either Oil red O, to visualize neutral lipids, or with a monoclonal antibody to intercellular adhesion

molecule 1 (ICAM-1) (Becton Dickinson) as previously described (2). Antibody visualization was performed by using a second incubation with a biotinylated rabbit anti-mouse IgG followed by an alkaline phosphatase-conjugated streptavidin and a red chromogen (Advanced Cellular Diagnostics, Elmhurst, Ill.).

Model building for structure predictions. An initial model for NRG1- β was built in analogy to the structure of human NDF (heregulin) (29) by using coordinates available from the Protein Data Bank (entry 1HRE) and the program Homology (MSI/Biosym, San Diego, Calif.). The coordinates of mouse EGF were similarly obtained from the database (entry 1EPI). The initial model was energy minimized with constraints on C α positions. The electrostatic potential was computed with the program Delphi (MSI/Biosym package), as has been previously described (22).

RESULTS

NRG isoforms transmit biological signals through distinct receptor combinations. While NRG1- β induces proliferation of many cell types, the factor promotes differentiation of certain mammary cell lines (2, 16, 44). Examination of the two NRG2 isoforms on AU-565 breast cancer cells indicated that both isoforms, like NRG1- β , can promote extensive morphological alterations, induce the appearance of vesicles containing neutral lipids, and up-regulate ICAM-1 (Fig. 1). These differentiation characteristics were shared with the other isoform of NRG1, NRG1- α , but its potency was significantly lower than that of the higher-affinity isoform, NRG1- β (data not shown). Likewise, dose-response analyses of the two NRG2 isoforms revealed that the α isoform was more active

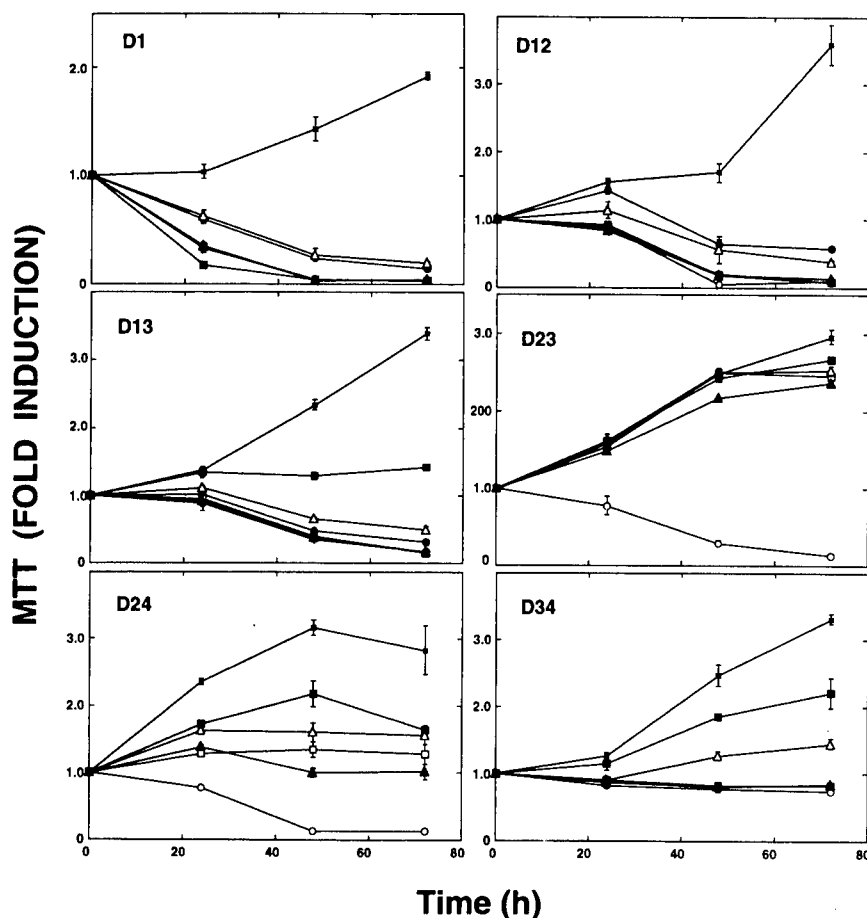


FIG. 3. Ligand-dependent survival of ErbB-expressing 32D cells in the absence of IL-3. The indicated sublines of 32D cells were incubated for various time intervals at a density of 5×10^5 cells/ml in the absence of IL-3 (open circles) or with one of the following ligands, each at a concentration of 100 ng/ml: EGF (solid circles), NRG1- α (open squares), NRG1- β (solid squares), NRG2- α (open triangle), or NRG2- β (solid triangle). For control, cells were incubated with medium conditioned by IL-3-producing cells (crosses). The extent of cell proliferation was determined daily by using the colorimetric MTT assay. The data presented are the mean \pm standard deviation of six determinations. The experiment was repeated twice with similar results.

than the β isoform of this family. For example, at a low concentration of NRG2- α (1 ng/ml), approximately 40% of treated cells displayed lipid vesicles, but a similar concentration of NRG2- β was practically inactive (20% positive cells). Taken together with the observation that NRG2- α can stimulate phosphorylation of ErbB-3 and ErbB-4 (7, 9, 12), the results presented in Fig. 1 suggested functional redundancy of the two NRG families.

To directly address this possibility, we performed comparative analysis of receptor specificity of the four NRG isoforms. An extended series of IL-3-dependent 32D myeloid cells that express individual ErbB receptors or their combinations (49) was used in conjunction with the MTT cell proliferation assay. These cells offer the advantage of receptor analysis in the absence of cross talk, because parental 32D cells express no known ErbB molecule. We have previously shown that the MTT assay reflects DNA synthesis and cell cycle progression in this particular cell system (48, 49). Out of the single ErbB-expressing cells, those expressing ErbB-2 alone (denoted D2 cells), as well as cells expressing the kinase-defective ErbB-3 protein alone (D3 cells), responded to no NRG isoform (Fig. 2). In contrast, D4 cells, which express ErbB-4 at relatively high levels, underwent enhanced proliferation in response to all four NRG isoforms (Fig. 2). Surprisingly, cells singly expressing ErbB-1 (D1 cells) responded to NRG2- α , but they

responded only weakly to very high concentrations of NRG2- β (Fig. 2). None of the two NRG1 isoforms was active on the ErbB-1-expressing 32D cells at concentrations as high as 100 ng/ml. In comparison with EGF, whose activity on D1 cells was detectable with as low a concentration as 0.1 ng/ml, the concentration of NRG2- α needed to elicit a similar response was at least 10-fold higher. While part of this discrepancy may be due to incomplete refolding of the synthetic NRG2 molecules we used, it is worthwhile noting that the NRG2- α -mediated effect exceeded, at high concentrations, the maximal response to EGF. In addition, long-term survival assays, which were performed with a single high dose of ligand, indicated that NRG2- α acted at least as efficiently as EGF in extending cell survival in the absence of IL-3 (Fig. 3). These observations, together with the specificity to NRG2- α , appear to attribute physiological relevance to the interaction between ErbB-1 and NRG2- α .

Examination of cell lines expressing various pairs of ErbB proteins revealed an overall isoform-specific pattern of dimer specificity: with all receptor combinations, NRG2- α was more potent than NRG2- β , whereas NRG1- β was superior to NRG1- α on cells expressing either ErbB-3 or ErbB-4 (Fig. 2 and 3). The relative potency, however, of the two more active NRG isoforms, NRG1- β and NRG2- α , displayed dimer dependency. For example, cells expressing a combination of

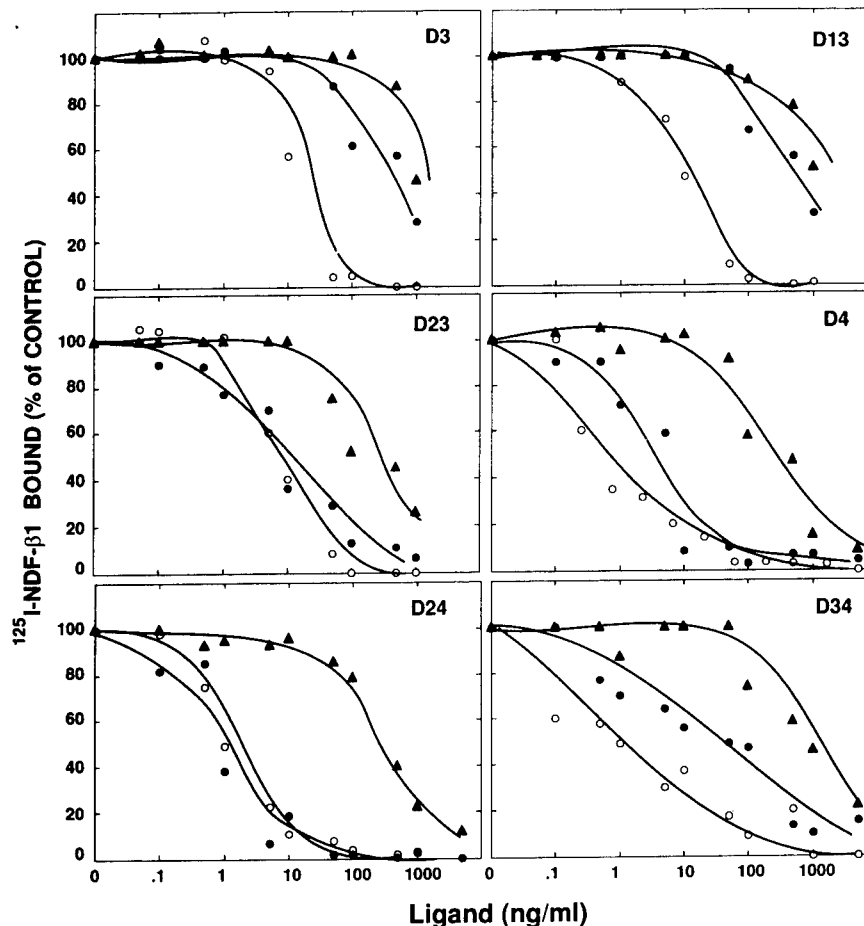


FIG. 4. Binding of type 2 neuregulins to specific ErbB proteins. Displacement analyses of radiolabeled NRG1- β were performed with the indicated derivatives of 32D cells. Cells (10^6) were incubated for 2 h at 4°C with the radiolabeled ligand (1 ng/ml) in the presence of increasing concentrations of an unlabeled NRG2- α (closed circles), NRG2- β (closed triangles), or NRG1- β (open circles). To remove unbound ligands, cells were sedimented ($12,000 \times g$, 2 min) through a cushion of calf serum at the end of the experiment, and their radioactivity was determined. Nonspecific binding of NRG1- β was determined in the presence of 100-fold excess of the unlabeled ligand. Each data point represents the mean (less than 10% variation) of two determinations.

ErbB-1 and ErbB-3 (D13 cells) were most efficiently stimulated by NRG1- β , which also acted as a potent survival factor for these cells (Fig. 3). D13 cells, however, responded to NRG2- α better than to EGF, and the two other NRG isoforms (NRG1- α and NRG2- β) were practically inactive (Fig. 2 and 3). A cooperative effect of ErbB-2 on binding (45, 55, 61) and cellular responses (23, 30, 49) to NRG1 has been previously described. This effect extends to NRG2 isoforms: coexpression of ErbB-2 and ErbB-3 sensitized cells to low concentrations of both types of NRG2 isoforms, and it also enhanced their potency to a level comparable to that of IL-3 (Fig. 2 and 3). In addition, the combination of ErbB-2 with ErbB-4 displayed remarkable sensitivity to NRG1- β and to NRG2- α (Fig. 2). For example, D34 cells that express ErbB-4 at the same level of D24 cells, but at least 10-fold lower than D4 cells, displayed significantly lower sensitivity to the more potent NRG isoforms (Fig. 2). In conclusion, the four NRG isoforms are distinct in their range of receptor specificity, and they collectively recognize all stimutable receptor combinations. Consequently, the resulting cellular responses display a graded pattern ranging from weak to potent mitogenicity (Fig. 2) and survival (Fig. 3).

Cooperative and isoform-specific recognition of ErbB proteins. Because previous comparison of the two NRG1 isoforms

revealed remarkable quantitative (60) and qualitative differences (48), it was interesting to analyze binding specificities and relative affinities of the two NRG2 isoforms and correlate them with the observed differences in biological response. First, we compared the capacity of each NRG2 isoform to displace a cell-bound radioactive NRG1- β . In line with the mitogenic superiority of the α isoform of NRG2, this type of isoform acted more efficiently than NRG2- β in the ligand displacement assay, on cells expressing all types of receptor combinations (Fig. 4). Similar to NRG1 isoforms, whose higher-affinity receptor is ErbB-4 (60), both types of NRG2s appear to bind to ErbB-4 with higher affinity than to the other receptor, ErbB-3 (compare D3 and D4 panels in Fig. 4). In agreement with the cooperative effect of ErbB-2, which was observed in both the cell proliferation assay and in the survival assay, coexpression of ErbB-2 together with ErbB-3 led to a 50-fold enhancement of NRG2- α affinity (Fig. 4). In fact, coexpression of ErbB-2 with ErbB-4 resulted in a greater affinity to NRG2- α than to NRG1- β , but the ErbB-4-ErbB-3 combination (D34 cells, Fig. 4) was not cooperative in terms of apparent ligand affinity.

Due to the relatively low affinity of NRG2 isoforms to ErbB-1, displacement of radiolabeled EGF from this receptor was inefficient (data not shown). Therefore, we used radiola-

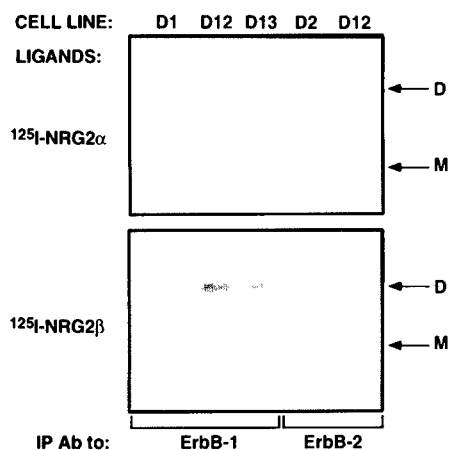


FIG. 5. Covalent cross-linking of radiolabeled NRG2 isoforms to ErbB-1-expressing cells. The indicated cells (10^7 cells per lane) expressing various ErbB proteins, including control cells expressing ErbB-2 alone (D2 cells), were incubated with either ^{125}I -NRG2- α or with ^{125}I -NRG2- β (each at 250 ng/ml). Following 90 min at 4°C , the covalent cross-linking reagent BS³ was added (1 mM, final concentration), and cell lysates were prepared after an additional 1.5 h of incubation. Affinity-labeled ErbB-1, ErbB-2, or ErbB-3 was immunoprecipitated by using specific mouse monoclonal antibodies, and the complexes were resolved by gel electrophoresis and autoradiography. Arrows mark the locations of monomeric (M) and dimeric (D) receptor complexes.

beled derivatives of NRG2 molecules and covalent cross-linking analysis to assay binding to ErbB-1 (Fig. 5). Evidently, both types of NRG2 molecules, when radiolabeled, displayed specific binding to monomers and dimers of ErbB-1. Presumably, NRG2- β binds to ErbB-1 with an affinity that is too low to allow mitogenicity (Fig. 2), but the procedure of covalent cross-linking makes this weak recognition detectable. Consistent with a cooperative effect, ErbB-2 specifically enhanced labeling of the dimeric form in D12 cells, although immunoprecipitation analysis implied that by itself ErbB-2 underwent only limited labeling by the radioactive ligand (Fig. 5). Specificity of labeling by NRG2s was evident by the absence of covalent cross-linking of ErbB-2, when singly expressed (D2 cells, Fig. 5), and by displacement with unlabeled EGF (data not shown). Taken together with the results of the displacement assay, our binding data support a model of isoform-specific pattern of receptor recognition.

Receptor phosphorylation and MAPK activation display distinct ligand-specific patterns. The remarkable differences we observed when comparing the actions of NRG isoforms in respect to cell proliferation and survival suggested that the distinct pairs of ligands and dimeric receptors differ in their signaling potencies. Indeed, comparisons of receptor phosphorylation on tyrosine residues were in line with the results obtained in the biological tests (Fig. 6). Whereas EGF stimulated extensive tyrosine phosphorylation of its receptor in D1 cells, the less-potent ligand, NRG2- α , induced a smaller effect, and the nonmitogenic ligand isoforms (NRG1s and NRG2- β) failed to stimulate tyrosine phosphorylation in these cells at a concentration of 100 ng/ml (Fig. 6A). In D13 cells, the most potent NRG isoform, NRG1- β , elicited higher tyrosine phosphorylation than the less potent NRG2- α isoform, while EGF was as effective as NRG1- β (Fig. 6A), probably because ErbB-1 expression exceeded the level of ErbB-3 in these cells. Examination of cells expressing various combinations of ErbB-2, ErbB-3, and ErbB-4 led to a similar conclusion, namely, that the extent of tyrosine phosphorylation of high-molecular-weight proteins, most likely activated ErbBs, corre-

lated with the relative mitogenic potency of NRG isoforms (Fig. 6B).

Because MAPKs are stimulated by all ligand-activated combinations of ErbB proteins (23, 30, 49), and they can integrate incoming signals (38, 54), we attempted to correlate the mitogenic potencies of NRGs with patterns of MAPK activation. Toward this end, we made use of a murine monoclonal antibody that specifically recognizes the active, doubly phosphorylated form of the ERK1 and ERK2 MAPKs (66). Immunoblotting of whole-cell lysates of D1 cells with this antibody revealed differences between the kinetics of MAPK activation by EGF and NRG2- α . In both cases, a delay of MAPK activation, compared to receptor phosphorylation, was observed, but receptor activation was more sustained with the more potent mitogen, EGF (Fig. 7A). Remarkably, the higher-molecular-weight form of MAPK, p44/ERK1, underwent activation only in response to EGF, and its kinetics were delayed. D4 cells, whose mitogenic responsiveness to NRGs was relatively high (Fig. 2), displayed relatively sustained and potent stimulation of MAPK (Fig. 7A), probably because these cells express approximately 10-fold more receptors than other derivative lines. Although the mitogenic action of the more potent NRGs, NRG1- β and NRG2- α , were comparable, (D4 panels in Fig. 2), MAPK activation was more prolonged in the case of NRG1- β , in agreement with the higher binding affinity of this ligand to ErbB-4 (Fig. 4). In D4, as well as in D23 cells, in which stimulation by NRGs was as potent as with IL-3 (Fig. 3), treatment with either NRG1- β or NRG2- α led to a robust and concomitant stimulation of both ERK1 and ERK2. Yet another pattern was shared by the two NRGs in D24 cells: both ERK isoforms were stimulated at the same early time point (1 min), but they, along with the receptors, displayed a relatively long decay (up to 120 min).

Analysis of MAPK activation by the relatively weak NRG isoforms, namely, NRG1- α and NRG2- β , extended the correlation with mitogenic activity and further supported the cooperative effect of ErbB-2 (Fig. 7B). Consistent with their weak or no mitogenic effect on D1 and D13 cells, the two isoforms induced practically no activation of MAPK in the two cell lines, but EGF was active in this assay. NRG1- α was more potent than NRG2- β on D4 cells, consistent with its higher mitoge-

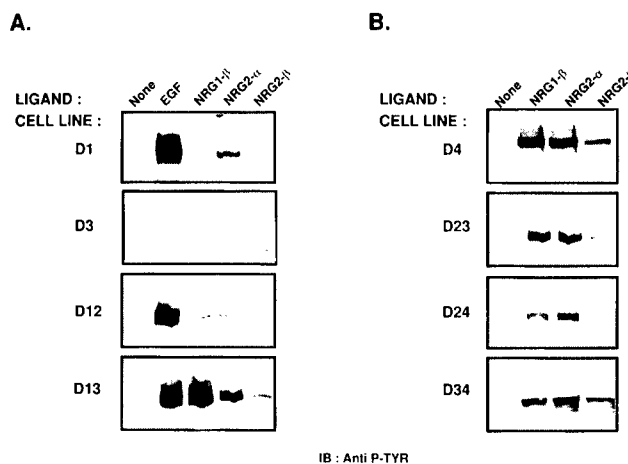
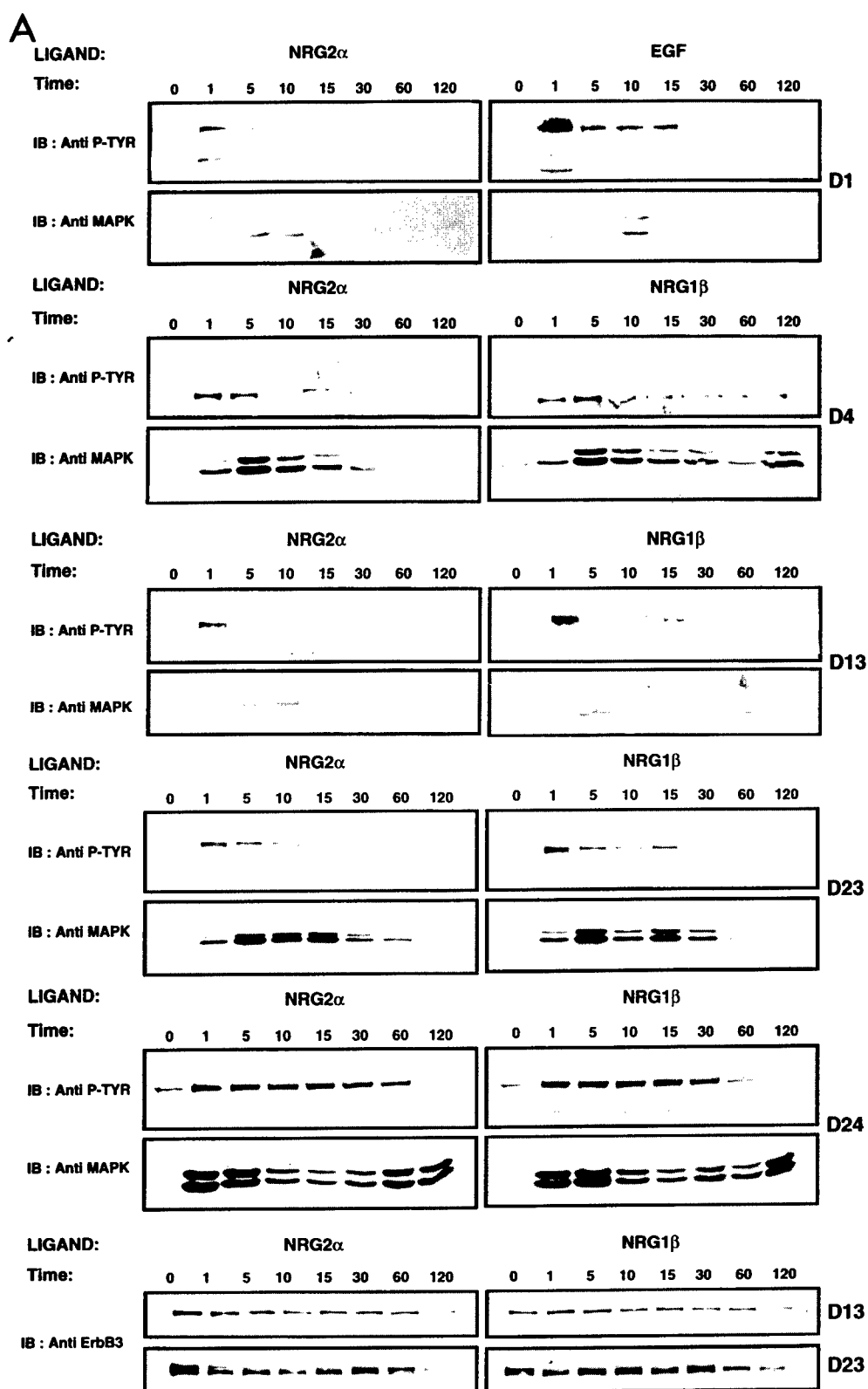


FIG. 6. NRG2-induced tyrosine phosphorylation of ErbB proteins. The indicated cell lines were incubated for 1 min at 37°C with either EGF, NRG1- α , NRG1- β , NRG2- α , or NRG2- β , each at 100 ng/ml. Control cultures were incubated with no added factor (None). Whole-cell lysates were then prepared, cleared from cell debris, and subjected to an immunoblot analysis with the PY-20 antiphosphotyrosine antibody. The regions of the gels corresponding to apparent molecular masses of 150 to 200 kDa are shown.



nicity for these cells. Finally, coexpression of ErbB-2, with either ErbB-3 or ErbB-4, significantly enhanced MAPK activation by the two relatively weak isoforms of NRG (Fig. 7B, D23 and D24). Taken together, the results presented in Fig. 7

indicate that the four isoforms of NRG, when acting through the four ErbB proteins, are able to set the MAPK pathway at different levels of activation, thus offering a basis for differences in biological potencies.

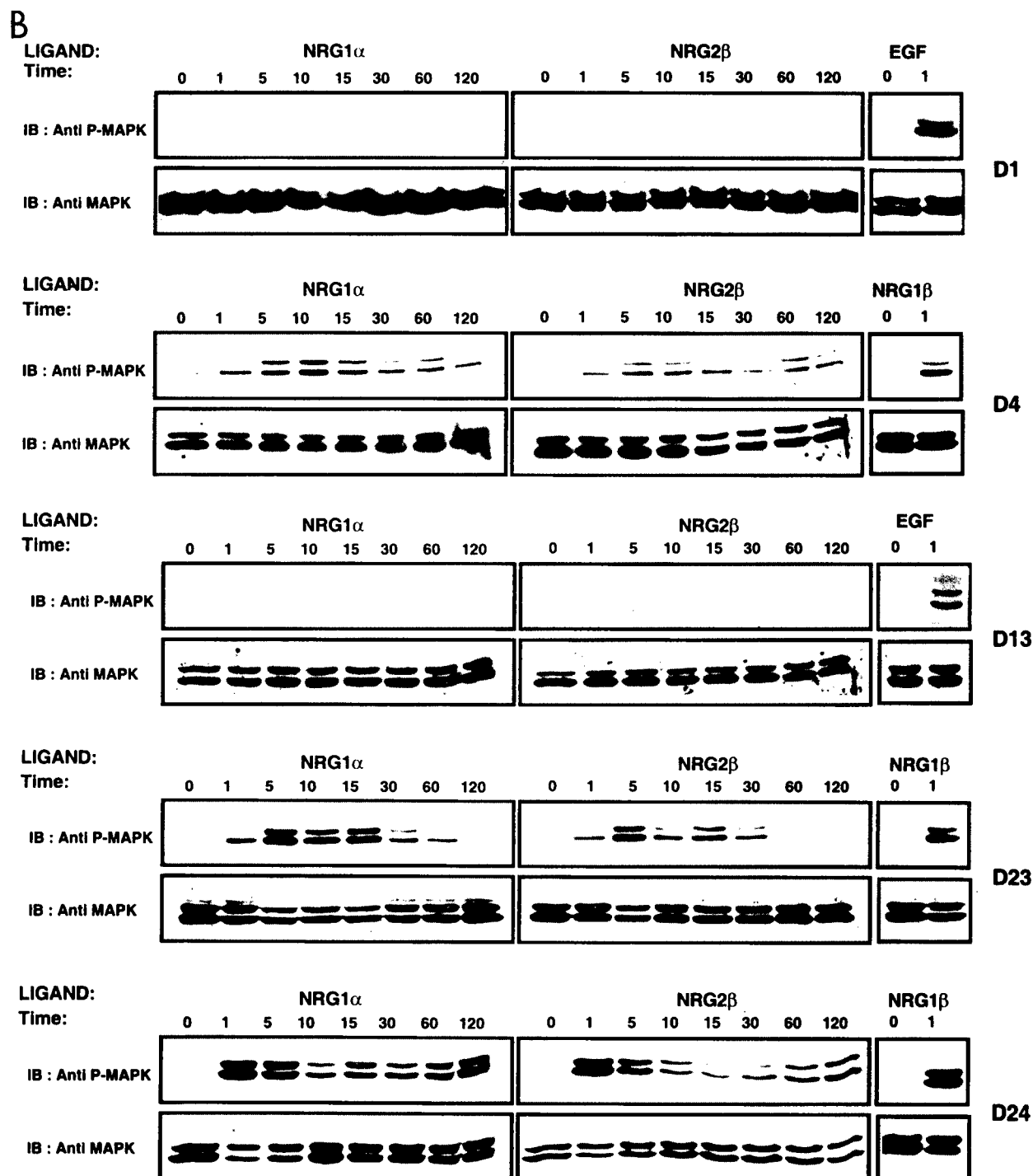


FIG. 7. Kinetics of receptor phosphorylation and MAPK activation by NRGs. The indicated derivatives of 32D cells were incubated for various time intervals (indicated in minutes) with growth factors (each at 100 ng/ml). All four isoforms of NRG1 and NRG2, along with EGF, were tested. Results obtained with the two more potent isoforms, NRG1- β and NRG2- α , are shown in panel A, and those obtained with the weaker factors, NRG1- α and NRG2- β , are shown in panel B. At the end of the incubation period, whole-cell lysates were prepared, cleared, and subjected to immunoblotting (IB) with either an antibody to phosphotyrosine (P-TYR) or with an antibody specific to the active doubly phosphorylated form of MAPK (66). Immunoblotting of whole-cell lysates with antibodies to ErbB-3 (A, bottom panels) or to the MAPK (B) were used to compare protein loading. Signal detection was performed by using a chemiluminescence kit.

DISCUSSION

Utilizing synthetic versions of the two newly reported NRG2 isoforms on a cellular system whose ErbB repertoire is defined, we identified a network of ligand-receptor interactions that is distinct from the one employed by NRG1 isoforms. Neverthe-

less, these two networks, which are schematically presented in Fig. 8, are partly overlapping and share several characteristics, including recruitment of ErbB-2 and its cooperative action, lack of interaction with homodimers of ErbB-2, and pairing of a relatively high-affinity ligand, whose range of receptors is

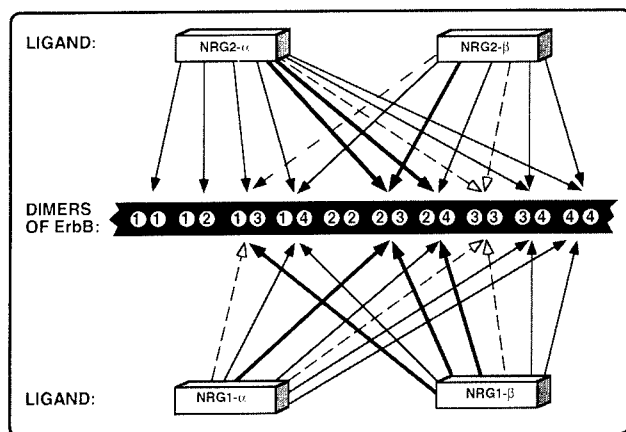


FIG. 8. Summary of ligand-receptor interactions within the NRG-ErbB signaling network. The horizontal gray bar represents the plasma membrane, and the various receptor combinations are shown schematically as circles. Specific ErbB proteins are identified by their numbers. The four major NRG isoforms are shown, and their strengths of signaling, as revealed by using the IL-3-dependent series of cell lines, are shown by arrows. Bold arrows indicate potent proliferative responses at low ligand concentrations (1 ng/ml or less). Note that no NRG isoform is able to activate the ErbB-3 homodimer (broken arrows), although all isoforms bind to this dimer. Likewise, NRG1- α cannot activate the ErbB-1/ErbB-3 heterodimer (48). In addition, no ligand binds to the ErbB-2 homodimer, but heterodimers of this protein with ErbB-3 or with ErbB-4 are relatively potent combinations. The information regarding the ErbB-1/ErbB-4 heterodimer was derived from Chinese hamster ovary cells overexpressing the two proteins (62). All other receptor combinations were examined in 32D cell derivatives.

broad (i.e., NRG1- β and NRG2- α), with a low-affinity ligand that binds to a relatively small set of dimeric ErbB combinations (NRG1- α and NRG2- β). Because spatial and temporal patterns of NRG1 expression are different from those exhibited by the more restricted NRG2 family (7, 9, 12), and the two isoforms of each family are expected to have yet their own distinct patterns (13, 40), the observed differences in receptor specificity are expected to increase functional diversity. Indeed, initial *in vitro* analyses of NRG1 and NRG2 revealed both quantitative and qualitative differences in activation of epithelial, muscle, and Schwann cells (6, 7).

It is worth noting that the structural difference between the α and β isoforms of NRG1, as well as NRG2 (Fig. 9A), is confined to the third loop of the EGF-like domain (loop C) and to the adjacent C terminus. This domain, however, is not the major site of structural variation, because the membrane proximal region, which connects the EGF-like domain of NRGs with the transmembrane stretch of proNRG molecules, displays broader variation (7, 27, 64). Whereas the juxtamembrane variation affects the rate of precursor processing, the more proximal heterogeneity, which represents alternative usage of one of two exons encoding the C-terminal loop of the EGF-like domain (7), critically influences receptor binding affinity (Fig. 4). The quantitative difference in affinity between NRG2 isoforms may translate into a qualitative one, since the analogous alteration in NRG1 dictates the differential ability of NRG1 isoforms to recruit ErbB-1 into a dimer with ErbB-3 (48). Likewise, the differences in receptor recognition displayed by the two direct ligands of ErbB-1, EGF and TGF α , are also due to a specific C-terminal sequence (34). In contrast, construction of hybrids between NRG1 and EGF revealed that the N terminus, rather than the C terminus, confers to NRG1 the ability to bind to its primary receptor (3). These observations can be explained by a model that attributes bivalence to NRG molecules (61). Accordingly, the N-terminal part of the

molecule allows high-affinity binding to a primary receptor, whereas the variant C-terminally located site confers an ability to recruit a secondary receptor. A bivalency model may apply also to EGF, because this ligand undergoes covalent cross-linking to different portions of ErbB-1, depending on whether cross-linking is mediated by the N or C terminus of EGF (58). In terms of bivalent ligand-receptor interactions, the broader and more potent signaling by NRG2- α is probably due to the C-terminally located binding site, whose affinity and range of ErbB specificity are larger than those of the corresponding site of NRG2- β .

Strikingly, all EGF-like ligands of ErbB proteins share very similar structures in their folded forms (29). This is dictated by the three-loop secondary structure and by a bilobular β structure that is held by hydrogen bonds. Interestingly, the middle loop of NRG1 (loop B, Cys2-Cys4) is longer by three amino acids than that of NRG2 (Fig. 9A). A similarly shorter loop exists in all ErbB-1-specific ligands, including EGF and TGF α . This structural feature may contribute to the ability of NRG2- α , but not NRG1s, to activate ErbB-1 in the absence of other ErbBs (Fig. 2 and 3). An alternative explanation is derived from the predicted folded structure of NRG2- α (Fig. 9B): although the compact structure of this ligand is in general similar to that of EGF and NRG1- β , the expected distribution of surface charges, especially in the C terminus, is more similar to that of EGF than to the practically neutral C tail of NRG1- β . In light of these considerations, it is worthwhile to address the question of why previous analyses did not detect interaction between NRG2 and ErbB-1 (7, 9, 12). Both Chang et al. (12) and Carraway et al. (9) used only the less potent isoform, NRG2- β , which is unable to stimulate ErbB-1 under normal conditions (Fig. 6A). Nevertheless, Carraway et al. (9) observed NRG2-induced ErbB-1 phosphorylation in MDA-MB468 cells, which express extremely high levels of ErbB-1. Possibly, ErbB-1 overexpression and the relatively high concentrations of recombinant NRG2- β used by these investigators enabled them to detect the weak interaction of NRG2- β with ErbB-1. Although, Busfield et al. used the higher-affinity ligand, NRG2- α (DON-1), none of their assays was aimed at detecting ErbB-1 activation. Apart from the interaction of NRG2- α with ErbB-1, our results are in full agreement with those of the three previous reports on NRG2. In fact, the observation that NRG1- β is more potent than NRG2- β in induction of epithelial cell flattening (12) and the evidence for better mitogenic response of mammary cells to NRG2- α than to NRG1- α (7) are consistent with the network we observed by using engineered myeloid cells (Fig. 8). Also consistent is the superiority of NRG1- β over NRG1- α in up-regulation of the acetylcholine receptor of chick muscle cells (7), but the complete inactivity of NRG2- α in this system may be attributed to a species barrier.

Our conclusion that each NRG isoform acts through a distinct set of dimeric receptors further extends the already large diversification potential of the ErbB signaling network (1). Three levels of diversity generation may be defined: In addition to the 10 dimeric receptor complexes, whose formation is ligand dependent and hierarchical (62), diversity is generated at the level of the multiple ligands, and more complexity is contributed by the many cytoplasmic signaling proteins that are recruited by each dimeric receptor complex. The ligand level exhibits remarkable diversity: Each ligand appears to differ from the others by its unique receptor specificity. Examples are betacellulin and the heparin-binding EGF-like growth factor, which bind to ErbB-4, in addition to ErbB-1 (18, 51) and EGF, an ErbB-1 ligand capable of activating the ErbB-2/ErbB-3 heterodimers at high concentrations (49a). Surpris-

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|----------------|--------|--------|----------|--------|--------|--------|--------|---------|
| | | | A | | | | | |
| NRG1- α | SHLVKC | AEKEK | AEKEK | AEKEK | AEKEK | AEKEK | AEKEK | CTENVPM |
| NRG1- β | SHLVKC | AEKEK | AEKEK | AEKEK | AEKEK | AEKEK | AEKEK | CQNYVMA |
| NRG2- α | GHARKC | NETAKS | NETAKS | NETAKS | NETAKS | NETAKS | NETAKS | CLEKLPL |
| NRG2- β | GHARKC | NETAKS | NETAKS | NETAKS | NETAKS | NETAKS | NETAKS | CQQFAMV |
| | | * | * | * | * | * | * | * |

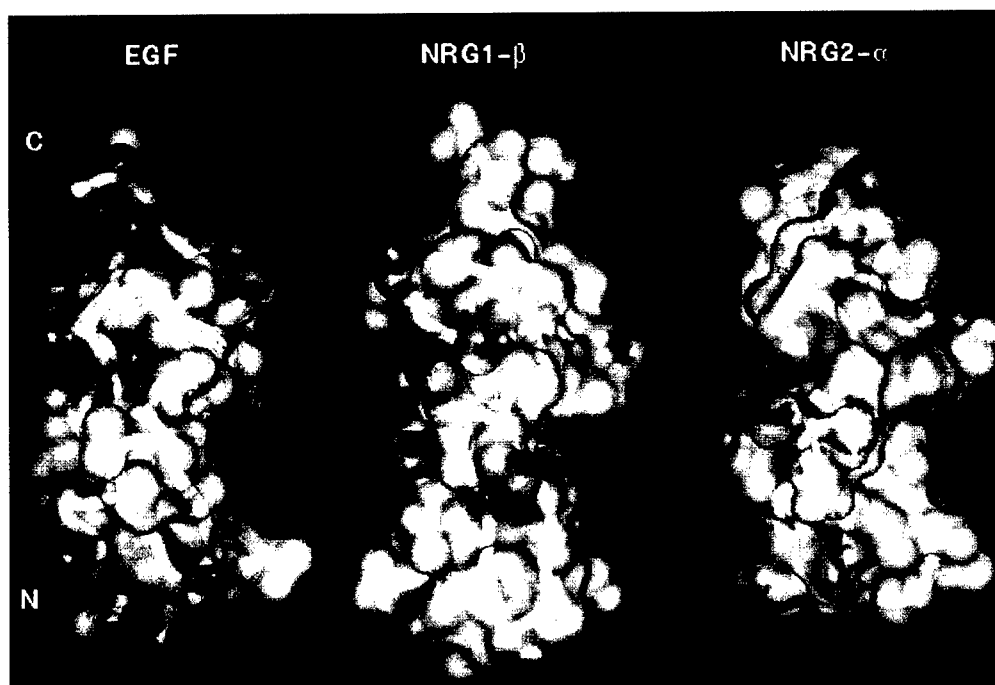
B

FIG. 9. Comparison of amino acid sequences and electrostatic potentials of three EGF-like ligands. (A) Alignment of amino acid sequences of the EGF-like domains of NRG1 and NRG2 isoforms. The three disulfide loops (A through C) are indicated, including the region shared by loops A and B (J region). Asterisks mark the canonical residues of the EGF-like family of ligands. (B) The figure depicts the solvent-accessible surfaces of the EGF-like domains of the molecules mouse EGF, rat NRG1- β , and rat NRG2- α . The molecules are colored according to their electrostatic potential: red for negative potential and blue for positive potential. Neutral areas are shown in white. The surfaces are transparent to show ribbon diagrams of the molecules (yellow). The locations of the N and C termini are indicated. Note the relatively extended structure of NRG-1 and its neutral C terminus. In contrast, the C termini of both EGF and NRG2- α are charged. Note that the N termini of the two types of NRG, a region that dictates high-affinity binding to ErbB-3 (3, 61), share a positive surface potential.

ingly, the third layer of signal diversification, namely, the effector molecules, displays only limited variation. Although each ErbB protein carries a distinct set of potential docking sites for cytoplasmic signaling proteins (10), only a few receptor-specific substrates have been actually identified. These include c-Cbl (36) and phospholipase C γ (15, 20), which are substrates of ErbB-1 and ErbB-2, but are unable to couple to ErbB-3 and ErbB-4. On the other hand, many signaling proteins, like Shc, Grb-2, and phosphatidylinositol 3' kinase (20, 50), are shared by the four ErbB molecules. Because we observed different patterns of MAPK activation upon cell stimulation with NRG2 (Fig. 7), and previous reports documented a similar phenomenon with other ligands, namely NRG1s and EGF (23, 30, 49), we raise an alternative mechanism of signal diversification at the effector level. Accordingly, specificity of

signaling is due to the variable degree of coupling to the MAPK pathway, rather than to an ErbB dimer-specific substrate(s). Thus, transient and weak activation of MAPK (especially ERK1) characterizes homodimers of ErbB-1, and sustained activation is observed with NRG-stimulated heterodimers of ErbB-2 with either ErbB-3 or with ErbB-4 (Fig. 7). The prolongation effect of ErbB-2 has been previously reported in mammary tumor cells and correlated with the extent of overexpression of this oncogenic protein (30). Conceivably, ErbB-2 prolongs NRG-mediated MAPK activation by its cooperative effect on ligand binding (Fig. 4). Additional factors that may extend MAPK activation are the relatively strong coupling of ErbB-2 to this pathway (5) and the uniquely slow rate of ErbB-2 endocytosis (56). Thus, the network of NRGs and ErbBs is able to translate the strength of ligand-receptor

interactions to different patterns of MAPK activation. This model is consistent with many results obtained in pheochromocytoma cells (PC-12), in which a correlation between the kinetics of MAPK activation and the type of cellular response, either proliferation or differentiation, was established (reviewed in reference 39). Finally, because only one ligand-ErbB pair exists in lower organisms, it is tempting to propose that the network of NRG and ErbB proteins represents a machinery developed throughout evolution for fine tuning of the MAPK pathway. Each of the multiple mammalian ErbB ligands may thus determine a specific setting of the ErbB module and consequently lead to cellular proliferation, survival, or differentiation. When fully active, like in the case of epithelial cells overexpressing ErbB-2 or maintaining NRG autocrine loops (for review, see reference 53), this pathway may contribute to cancer development.

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Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers

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Both homo- and hetero-dimers of ErbB receptor tyrosine kinases mediate signaling by a large group of epidermal growth factor (EGF)-like ligands. However, some ligands are more potent than others, although they bind to the same direct receptor. In addition, signaling by receptor heterodimers is superior to homodimers. We addressed the mechanism underlying these two features of signal tuning by using three ligands: EGF; transforming growth factor α (TGF α); and their chimera, denoted E4T, which act on cells singly expressing ErbB-1 as a weak, a strong, and a very strong agonist, respectively. Co-expression of ErbB-2, a developmentally important co-receptor whose expression is frequently elevated in human cancers, specifically potentiated EGF signaling to the level achieved by TGF α , an effect that was partially mimicked by ErbB-3. Analysis of the mechanism underlying this *trans*-potentiation implied that EGF-driven homodimers of ErbB-1 are destined for intracellular degradation, whereas the corresponding heterodimers with ErbB-2 or with ErbB-3, dissociate in the early endosome. As a consequence, in the presence of either co-receptor, ErbB-1 is recycled to the cell surface and its signaling is enhanced. This latter route is followed by TGF α -driven homodimers of ErbB-1, and also by E4T-bound receptors, whose signaling is further enhanced by repeated cycles of binding and dissociation from the receptors. We conclude that alternative endocytic routes of homo- and hetero-dimeric receptor complexes may contribute to tuning and diversification of signal transduction. In addition, the ability of ErbB-2 to shunt ligand-activated receptors to recycling may explain, in part, its oncogenic potential.

Keywords: endocytosis/ErbB/HER family/oncogene/
signal transduction/transforming growth factor α

transmembrane allosteric kinases with specificity to tyrosine residues (van der Geer *et al.*, 1994). As in other allosteric systems, the monomeric form of the receptor tyrosine kinase (RTK) is inactive, but upon ligand-induced oligomerization (primarily dimerization) it initiates a plethora of intracellular events ranging from stimulation of ion fluxes to cytoskeletal alterations, and culminating in regulation of gene expression. The underlying biochemical mechanism involves autophosphorylation of specific tyrosine residues of the activated receptor. These are turned into docking sites for cytoplasmic signaling proteins containing Src-homology 2 (SH-2) domains (Koch *et al.*, 1991), such as the adapter molecules SHC, Sem-5/Grb-2 and the p85 subunit of phosphatidylinositol 3' kinase (Eagan and Weinberg, 1993). As a consequence thereof, several linear cascades of protein kinases are triggered, including the mitogen-activated protein kinase (MAPK) pathway (Seger and Krebs, 1995) and the S6-kinase pathway (Ming *et al.*, 1994).

In addition to this 'vertical' transduction pathway, lateral propagation of growth factor signals is made possible within subgroups of homologous RTKs by means of receptor heterodimerization. The best characterized example of 'lateral' signaling is provided by the type I RTKs (also named ErbB or HER family) (Carraway and Cantley, 1994; Alroy and Yarden, 1997). This subfamily comprises four members whose prototype is ErbB-1, a receptor that binds several ligands, including epidermal growth factor (EGF) and transforming growth factor (TGF α). Likewise, ErbB-3 and ErbB-4 bind three groups of alternatively spliced growth factors, collectively called neuregulins (Burden and Yarden, 1997). The fourth member, ErbB-2, binds no known ligand with high affinity. Nevertheless, impairment of ErbB-2 function by gene targeting resulted in a phenotype shared with that of neuregulin- and ErbB-4-deficient embryos (Lee *et al.*, 1995), and a mutant form of this receptor promotes cancer in rodents (Bargmann *et al.*, 1986). Overexpression of the wild-type human protein leads to phenotypic transformation of cultured cells (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987), and is frequently observed in several types of human carcinomas (Slamon *et al.*, 1987, 1989). Moreover, ErbB-2 overexpression predicts poor prognosis and resistance to certain therapeutic modalities, implying that the orphan receptor contributes to tumor virulence (reviewed in Hynes and Stern, 1994; Stancovski *et al.*, 1994). Despite the absence of a direct ligand, ErbB-2 plays a central role in a network of inter-receptor interactions; although the four ErbBs can form all 10 possible homo- and hetero-dimeric combinations, ErbB-2-containing heterodimers are preferred over other combinations (Tzahar *et al.*, 1996; Graus-Porta *et al.*, 1997). Each dimeric receptor complex has a distinct signaling potency, resulting in diversification and fine-tuning of signaling (Riese *et al.*, 1995; Pinkas-

Introduction

A large group of polypeptide growth factors mediates intercellular signaling by binding to, and activation of,

Kramarski *et al.*, 1996a). In general, signaling by homodimeric complexes is relatively weak, whereas heterodimers, and especially those containing ErbB-2, are more potent transmitters of signals. The collaborative action of two different ErbBs is best exemplified by the potent combination of ErbB-2, the ligandless receptor, with ErbB-3, whose kinase function is defective, and is reflected by the synergistic effect on cell transformation of certain co-expressed pairs of ErbBs (Kokai *et al.*, 1989; Alimandi *et al.*, 1995; Wallasch *et al.*, 1995).

In addition to the receptor level, combinatorial signaling by the ErbB network is further diversified at two additional levels. First, multiple EGF-like ligands exist and they differentially induce certain receptor combinations (Pinkas-Kramarski *et al.*, 1996b), probably because each ligand carries not only a high affinity site, but also a 'low affinity/broad specificity' site that recruits the dimer's partner (Tzahar *et al.*, 1997). Interestingly, some ligands induce more potent signals than others although they bind to the same receptor. For example, on certain cellular systems, such as keratinocytes (Barrandon and Green, 1987) and endothelial cells (Schreiber *et al.*, 1986), TGF α is more potent than EGF, although both ligands bind to ErbB-1 with comparable affinity (Kramer *et al.*, 1994). Another level of signal diversification is comprised of the multiple substrates of RTKs; members of this large group of SH-2 domain-containing proteins are differentially recruited to certain ErbBs. Examples include the phosphatidylinositol 3'-kinase and c-Cbl that preferentially engage with ErbB-3 (Soltoff *et al.*, 1994) and with ErbB-1 (Levkowitz *et al.*, 1996), respectively. Despite differences in second messenger activation, signaling by all ErbBs feeds into the MAPK pathway, raising the question of how signal specificity is maintained intracellularly. One potential answer is provided by results obtained with other growth factors in pheochromocytoma cells, indicating that the kinetics of MAPK activation, and especially its inactivation, may critically determine signal identity (reviewed in Marshall, 1995). Unlike the activation process which has been extensively studied, the inactivation phase of RTK signaling is poorly understood. One obvious candidate is the process that leads to endocytosis, down-regulation and degradation of ligand-activated receptors. Indeed, individual ErbB proteins differ remarkably in their rate of endocytosis and down-regulation (Baulida *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996a).

Our present study addressed the hypothesis that the multiple ligands of ErbBs differ in their potencies because they differentially recruit certain heterodimeric receptor combinations (Beerli and Hynes, 1996; Pinkas-Kramarski *et al.*, 1996b; Gulliford *et al.*, 1997). To this end we compared signaling by EGF and TGF α , a pair of ligands that display respectively weak and strong signaling in most tissues, in a well-defined cellular system expressing combinations of ErbB-1 with either ErbB-2 or ErbB-3. In contrast to our working hypothesis, differences in potency were observed even in the absence of either co-receptor, namely ErbB-2 or ErbB-3. However, to our surprise, the co-receptors potentiated the effect of EGF without significantly affecting TGF α signaling. In subsequent experiments we investigated the mechanism of potentiation and found that the co-receptors, by forming heterodimers with ErbB-1, redirected this receptor to an endocytic route

that allows receptor recycling and, therefore, enhanced signaling. These results imply that EGF-like ligands whose ErbB specificity is shared are functionally distinct, and suggest that alternative endocytic routing may be critical for controlled inactivation and fine-tuning of signal transduction.

Results

ErbB-2 and ErbB-3 potentiate EGF mitogenicity but not TGF α signaling

To examine possible functional relationships between the multiplicity of EGF-like ligands and the extensive inter-receptor interactions within the ErbB family of receptors we used the two best characterized ligands of the family, namely EGF and TGF α , in combination with a series of cell lines co-expressing ErbB-1 with either ErbB-2 (D12 cells), or with ErbB-3 (D13 cells) (Pinkas-Kramarski *et al.*, 1996a). A third cell line that singly expresses ErbB-1 (D1 cells) was used for comparison of ErbB-1 homodimers with heterodimers of this receptor. In addition, a chimeric EGF/TGF α molecule, designated E4T, comprised of the A and B loops of EGF, and the C loop of TGF α , was used because of its superior mitogenic activity to that of other chimeric molecules and the parental ligands (Lenferink *et al.*, 1997). Due to their dependence on interleukin-3 (IL-3), the cell lines we employed are extremely sensitive to EGF-like ligands when tested in the absence of IL-3. Thus, TGF α exerted mitogenic stimuli that were at least 10-fold more active than EGF-induced signals when tested on D1 cells (Figure 1A). However, E4T was even more potent in inducing cell proliferation. This pattern of relative potency was also reflected in long-term survival experiments in which IL-3 was replaced by the corresponding ErbB-1 ligand and cell survival monitored daily (Figure 1B). Introduction of ErbB-2 into D1 cells elevated the basal proliferation rate of the resulting cell line, D12, in agreement with previous reports (Kokai *et al.*, 1989; Cohen *et al.*, 1996; Tzahar *et al.*, 1996; Zhang *et al.*, 1996). Thus, whereas maximal stimulation of D1 cells by IL-3 was 5.5-fold, only a 2-fold activation was displayed by D12 cells. Interestingly, however, co-expression of ErbB-2 together with ErbB-1 (D12 cells) resulted in remarkable potentiation of the mitogenic action of EGF; whereas half maximal mitogenic effect was induced by 10 ng/ml of this ligand on D1 cells, only 0.7 ng/ml was necessary to stimulate the D12 cells (Figure 1A, compare D1 with D12 panels). In contrast, ErbB-2 co-expression only slightly improved the mitogenic action of TGF α and E4T. In fact, in the presence of ErbB-2, EGF almost approached the high mitogenic activity of TGF α , a phenomenon that was reflected, in part, also in a long-term survival assay (Figure 1B, D12 panel). Interestingly, ErbB-3 only partially potentiated EGF activity in D13 cells (compare the EC₅₀ of EGF on D13 cells, which is 2 ng/ml, with that on D1 cells, which is 10 ng/ml). Once again, co-expression exerted no significant effect on the potency of either TGF α , or E4T (D13 panels in Figure 1). In conclusion, ErbB-2, and to some extent also ErbB-3, specifically enhance the EGF-induced mitogenic action of ErbB-1, probably by forming heterodimeric complexes with this receptor.

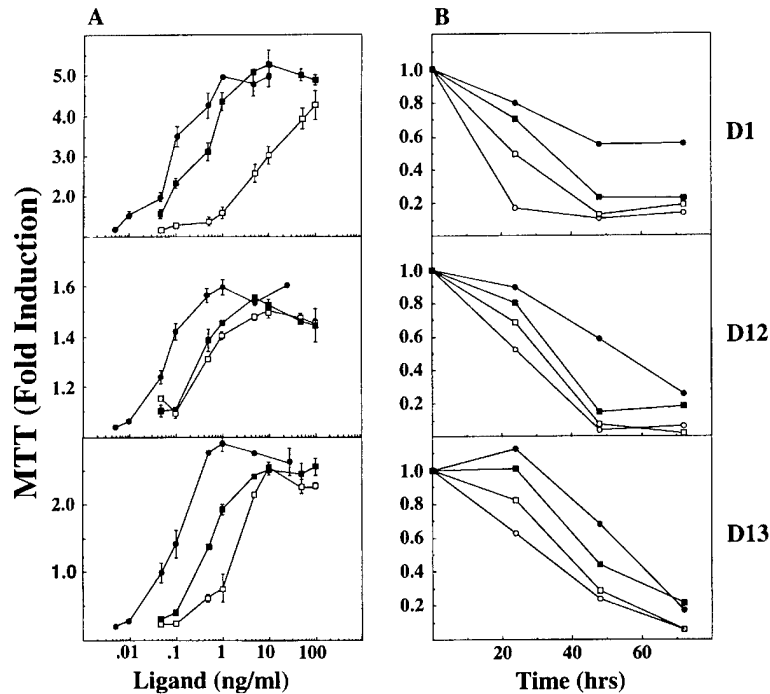


Fig. 1. Ligand-induced proliferation and survival of ErbB-expressing 32D-cells. (A) The following derivatives of 32D cells were examined for cell proliferation by using the MTT assay: D1 cells that singly express ErbB-1, D12 cells expressing a combination of ErbB-1 with ErbB-2, and D13 cells expressing a combination of ErbB-1 with ErbB-3. Cells were washed free of serum factors and IL-3, and seeded at a density of 5×10^5 cells/ml in RPMI-1640 medium containing serial dilutions of EGF (\square), TGF α (\blacksquare), or E4T (\bullet). Following 24 h of incubation, the MTT assay was performed as described in Materials and methods. (B) The indicated sublines of 32D-cells were plated as described above in the presence of 100 ng/ml EGF, TGF α or E4T [symbols are as in (A)]. Cell proliferation was measured daily using the MTT assay. As a negative control cells were plated in serum- and IL-3-free medium (\circ). The data from both experiments are given as the means of three determinations. Bars in (A) represent standard deviations. The experiments were repeated three times. The responses to IL-3 (fold induction) of D1, D12 and D13 were 5.54 ± 0.63 , 1.96 ± 0.67 and 3.03 ± 0.81 , respectively.

Binding parameters may explain superiority of E4T, but not the difference between EGF and TGF α

Perhaps the simplest explanation for the observed differences in mitogenic potencies of EGF, TGF α and E4T might be parallel differences in receptor binding affinities. To examine this possibility we labeled the three ligands with ^{125}I and determined their apparent binding affinities to D1, D12 and D13 cells using ligand displacement analysis. The results of this experiment are shown in Figure 2A. Evidently, the apparent affinities of EGF, TGF α and E4T were not remarkably different when tested on D1 cells, in agreement with a similar analysis that was performed with fibroblasts (Lenferink *et al.*, 1997). Co-expression of ErbB-2 (or ErbB-3) only slightly improved the affinity of D12 cells (or D13 cells) to EGF or TGF α (Figure 2A, D12 and D13 panels). Notably, ligand binding assays performed with derivatives of 32D cells usually yield affinities that are consistently lower than those measured with adherent cell types such as fibroblasts or epithelial cells. For example, the K_d values of EGF and TGF α binding to adherent cells are in the range of 0.1–5 nM (Tzahar *et al.*, 1994; Lenferink *et al.*, 1997), whereas D1 cells bind these ligands with apparent K_d values of 30–50 nM. This may be due to the relatively prolonged washing procedure required in the case of the 32D myeloid cells, which results in an overall reduction in assay sensitivity. We used a ligand dissociation assay as an alternative to partly overcome this limitation. Cells were loaded with the various radiolabeled ligands under saturating conditions, then the unbound ligand was removed and

the rates of release of radioactivity were monitored. Clearly, the rates of release of E4T from the surfaces of all three cell lines examined were higher than the dissociation rates of EGF and TGF α (Figure 2B). In addition, the co-expressed co-receptors, namely ErbB-2 and ErbB-3, comparably decelerated the rate of dissociation of EGF and TGF α from ErbB-1, in agreement with previous reports (Kokai *et al.*, 1989; Karunakaran *et al.*, 1996; Tzahar *et al.*, 1996). Taken together, rapid dissociation from the cell surface may be involved in the mitogenic superiority of E4T over EGF and TGF α . However, neither the enhancement of EGF signaling by the co-receptor, nor the superiority of TGF α over EGF may be attributed to binding parameters.

Co-receptors decelerate ligand depletion and internalization, but clearance of the E4T superagonist is defective

Because E4T is released from the cell surface at a much faster rate than EGF or TGF α , we expected that these latter ligands would be depleted from the medium at a much faster rate than E4T. This possibility was tested by incubating D1, D12 and D13 cells with serial dilutions of the ligands for 24 h, thereby allowing their depletion from the medium. Then we determined the relative concentration of each ligand in the conditioned medium by employing a bioassay that uses serum-starved HER-14 fibroblasts overexpressing ErbB-1. As predicted, the rate of ligand depletion inversely correlated with mitogenic potency; the weakest and the strongest mitogens of D1 cells, namely

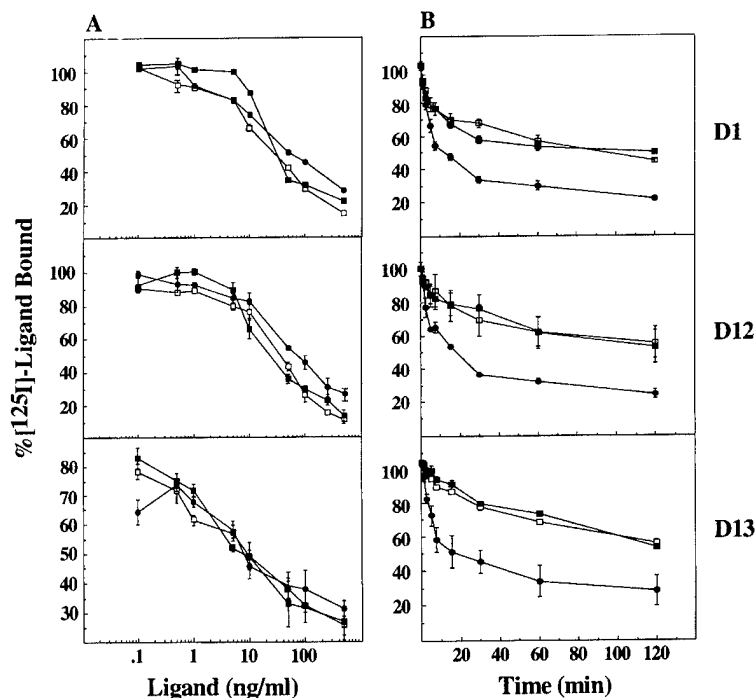


Fig. 2. Ligand displacement and dissociation analyses. (A) Displacement analysis was performed with 1.0×10^6 cells of the indicated subclones of the 32D cell line. Cells were washed free of IL-3 and serum factors using binding buffer, and subsequently incubated for 2 h at 4°C with [¹²⁵I]EGF (1 ng/ml) in the presence of serial dilutions of unlabeled EGF (□), TGFα (■) or E4T (●). Unbound ligand was removed by sedimenting the cells through a cushion of calf serum. The results are presented as the mean \pm SD of two determinations. Experiments were repeated three times with similar results. (B) The indicated cell lines were incubated for 2 h at 4°C with [¹²⁵I]EGF (□), [¹²⁵I]TGFα (■), or [¹²⁵I]E4T (●), each at 60 ng/ml. Then, the unbound ligand was replaced by an excess of the unlabeled growth factor (3 μ g/ml), and cell-bound radioactivity was monitored at the indicated time intervals. Results are expressed as the fractional ligand binding (mean \pm SD) relative to the amount of ligand that bound at $t=0$. The experiment was performed in duplicate and repeated twice with similar results.

EGF and E4T, respectively displayed rapid and slow depletion from the medium (Figure 3A). For example, when D1, D12 and D13 cells were incubated for 24 h with a low concentration of EGF (1 ng/ml) and the resulting conditioned media compared with medium similarly incubated in the absence of cells, we observed a 63, 28 and 47% reduction, respectively, in mitogenic activity. The corresponding numbers for TGFα were 28, 36 and 43%, and for E4T, 14, 16 and 24%. Thus, the presence of ErbB-2 significantly decelerated the rate of EGF depletion, but it less efficiently affected removal of E4T or TGFα from the medium. The relative rates of cell-mediated removal of the three ligands correlated with their mitogenic potency, implying that an endocytic mechanism is responsible for the observed differences in signaling potency. Consistent with this model, co-expression of the less potent co-receptor, ErbB-3, together with ErbB-1 only partly extended the half life of EGF (D13 panel in Figure 3A).

To test directly a model involving endocytosis, we comparatively analyzed the internalization rates of the various ligands of ErbB-1, and also determined their dependence on the presence of a co-receptor, either ErbB-2 or ErbB-3. It is notable that our previous experiments, which used a standard ligand internalization assay, detected only minor differences between the rates of ligand internalization through homo- and hetero-dimeric receptors (Pinkas-Kramarski *et al.*, 1996a). Therefore, we tested several ligand internalization protocols for their ability to discriminate between the rates of endocytosis of homo- and hetero-dimeric receptors and selected the following

assay. Cells were first incubated in the cold with a moderately low concentration of the respective radio-labeled ligand, then the unbound ligand was removed, cells chased at 37°C with a saturating ligand concentration and the ligand distribution between the cell surface and the cytoplasm was determined using an acid wash. This protocol differs from that previously employed (Pinkas-Kramarski *et al.*, 1996a) in two aspects. First, a 10-fold lower ligand concentration was used in order to avoid saturation of the coated pit-mediated internalization pathway (reviewed in Sorkin and Waters, 1993). Secondly, other protocols do not include a step that removes unbound ligand prior to initiation of endocytosis. Therefore, continuous uptake of the radiolabeled ligand may mask differences in endocytosis rates. The results of this experiment presented in Figure 3B confirmed that internalization of E4T is significantly slower than that of EGF or TGFα. More importantly, the rate of EGF uptake was remarkably decelerated by a co-expressed ErbB-2, but less so in the presence of ErbB-3 (EGF panel in Figure 3B). The rate of TGFα internalization was similarly affected by the presence of ErbB-2 or ErbB-3 (hTGFα panel in Figure 3B), implying that receptor heterodimers endocytose more slowly than homodimers, irrespective of ligand identity. Because both homodimers and heterodimers of ErbB-1 apparently exist in D12 and in D13 cells, the net kinetics of heterodimer internalization is expected to be even slower than the rates reflected in Figure 3B. Taken together, the data presented in Figure 3 suggest that signaling superiority of E4T is due to the slow rates of internalization and clearance of this ligand from the medium. Possibly,

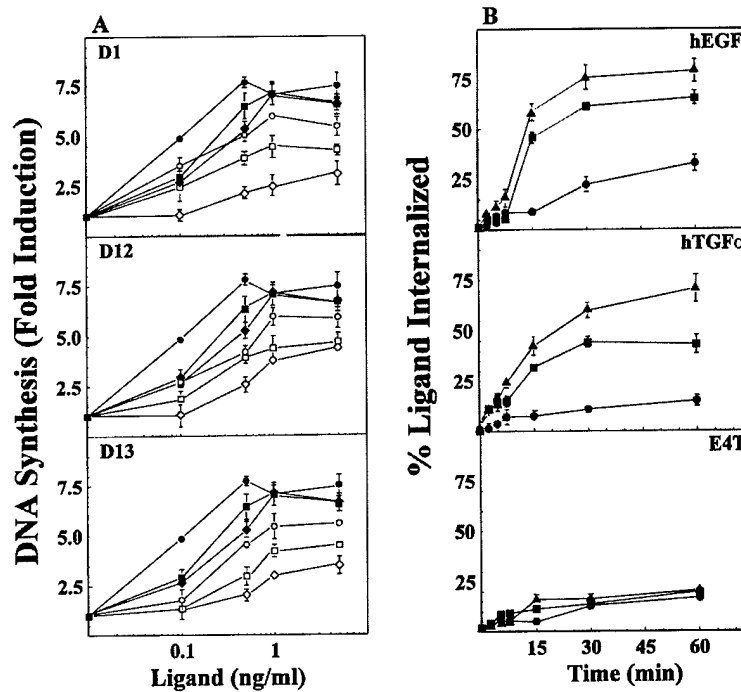


Fig. 3. Receptor-mediated depletion and uptake of ligands. (A) Increasing concentrations of the following ligands were incubated for 24 h at 37°C with the indicated derivatives of 32D cells (open symbols): EGF (diamonds), TGFα (squares) or E4T (circles). For control, ligands were similarly incubated in the absence of cells (closed symbols). The capacity of the resulting conditioned media to stimulate DNA synthesis in HER-14 fibroblasts was then determined as described in Materials and methods. Results are given as the mean \pm SD of three individual experiments carried out in duplicate. (B) For determination of ligand internalization rates, radiolabeled forms of the indicated ligands (each at 1 ng/ml) were incubated for 2 h at 4°C with the following derivatives of 32D cells: D1 (\blacktriangle), D12 (\bullet) or D13 cells (\blacksquare). Following incubation on ice, cells were washed free of unbound ligand and incubated at 37°C for various time intervals with excess of the corresponding unlabeled ligand (at 3 μ g/ml). Cellular uptake of radioactivity was monitored by removing surface-bound ligand with an acidic ligand-strip buffer. Data are presented as the mean \pm SD of duplicate determinations. Each experiment was repeated at least twice.

rapid dissociation of E4T from ErbB-1 (Figure 2) prevents efficient internalization. On the other hand, the relatively weak signaling capacity of EGF through the singly expressed ErbB-1 is attributed by our results to the efficient rate of cellular uptake of this ligand. Moreover, the potentiating effect of ErbB-2 is probably due to its ability to decelerate both the rate of internalization (Figure 3B) and the rate of clearance of EGF from the medium (Figure 3A), in line with the relatively slow down-regulation and endocytosis of ErbB-2 (Sorkin *et al.*, 1993; Baulida *et al.*, 1996). Despite these consistencies, our results cannot provide a satisfactory explanation for the relatively high potency of TGFα; although this ligand is more potent than EGF on D1 cells, and it is almost equipotent to EGF on D12 cells (Figure 1), its rates of internalization (Figure 3B), depletion from the medium (Figure 3A) and dissociation from the cell surface (Figure 2B), are only slightly different than those of EGF, and they apparently cannot account for the EGF-specific 10–15-fold mitogenic enhancement effect of ErbB-2 (Figure 1A).

EGF and TGFα are comparably degraded, but E4T degradation is limited

According to one possibility, EGF and TGFα are similarly endocytosed, but whereas the former is efficiently degraded in lysosomes, the other escapes intracellular degradation. To test this model we treated cells with each of the radiolabeled ligands under conditions that prevent receptor recycling and retard targeting to the degradative pathway. Upon transfer of chilled cells to 37°C ligand degradation

was allowed and monitored using acid precipitation. The results presented in Figure 4 indicate that E4T is degraded at a slower rate than EGF and TGFα, as expected on the basis of its slower rate of uptake (Figure 3B), but intracellular degradation of EGF and TGFα were comparable in kinetics and extent. Remarkably, expression of a co-receptor together with ErbB-1 only slightly affected the rates of ligand degradation. In experiments not shown we confirmed a previous report (Hamel *et al.*, 1997) that degradation of both ligands was significantly inhibited by chloroquine, a drug known to inhibit degradation in both endosomal (prelysosomal) and lysosomal compartments, but leupeptin, a tripeptide whose inhibitory action is specific to lysosomes (Cardelli *et al.*, 1989), did not affect TGFα degradation. Conceivably, EGF is destined for lysosomal degradation after endocytosis (Renfrew and Hubbard, 1991), whereas TGFα is degraded in a non-lysosomal compartment whose identity is only partly characterized (Hamel *et al.*, 1997). Independent of its exact intracellular location, endocytic degradation of EGF and TGFα cannot provide an explanation for the superiority of TGFα and the potentiating effect of ErbB-2.

The presence of a co-receptor specifically increases acid sensitivity of EGF binding

It is well established that binding of EGF and TGFα (Ebner and Derynck, 1991), as well as binding of various chimeras of these two ligands (Lenferink *et al.*, 1997), display differential sensitivity to acidic pH. This, in turn, is thought to allow recycling of TGFα-bound receptors to

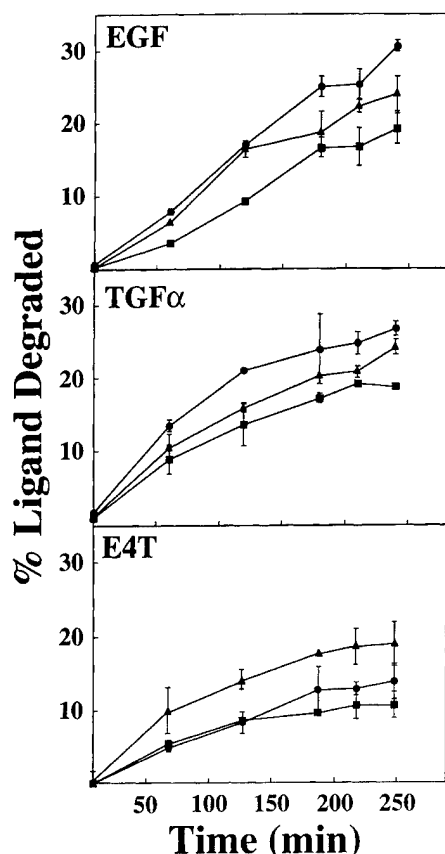


Fig. 4. Kinetics of ErbB-mediated ligand degradation. The indicated radiolabeled ligands (each at 1 nM) were incubated for 1 h at 20°C with the following derivatives of 32D cells: D1 (▲), D12 (●) or D13 cells (■). Thereafter, the cells were spun through a cushion of serum to remove unbound ligand, and then incubated at 37°C for various time intervals. Media were then collected and cells solubilized. The fraction of acid-soluble (degraded) ligand in the medium was determined by counting the acid-soluble radioactivity in the medium and the total cell-associated radioactivity. The results are expressed as the average percentage of acid-soluble radioactivity, relative to the sum of cell-associated and medium-released radioactive counts. Bars represent standard deviations. The experiment was performed in duplicate and repeated twice.

the cell surface, thereby augmenting TGF α biological action (Ebner and Derynck, 1991). On the other hand, because EGF resists the moderately acidic pH of early endosomes, this ligand does not permit receptor recycling, and the ligand-receptor complex is destined for degradation in lysosomes. To examine the possibility that the presence of a co-receptor alters pH sensitivity of ligand binding, we analyzed the interaction between EGF, TGF α and E4T with D1, D12 and D13 cells under various pH conditions. In line with previous observations, EGF binding to ErbB-1 displayed remarkable stability when compared with TGF α and E4T (Figure 5). However, the presence of a co-receptor, either ErbB-2 or ErbB-3, significantly destabilized these interactions. By contrast, the co-receptors only slightly affected the relatively sensitive binding of TGF α (hTGF α panel in Figure 5). In addition, a moderate effect of the co-receptors was observed in the case of E4T (Figure 5). On the basis of these observations we predict that the lysosome-destined EGF-driven ErbB-1 is re-routed to recycling back to the

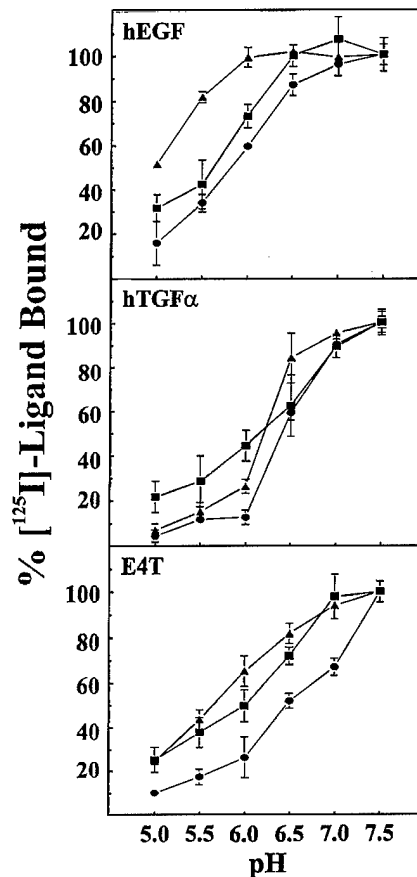


Fig. 5. pH sensitivity of ligand binding to specific combinations of ErbBs. D1 (▲), D12 (●) or D13 cells (■) were incubated for 2 h at 4°C with radiolabeled forms of the indicated ligands (each at 60 ng/ml). The pH of the binding buffer was adjusted to the indicated values. Unbound radioactivity was removed by sedimenting the cells through a cushion of calf serum, prior to γ -counting. Results are shown as the mean \pm SD of a triplicate experiment which was repeated twice.

cell surface once a co-receptor is present. On the other hand, co-expression of ErbB-2 or ErbB-3 may not alter routing of a TGF α -driven ErbB-1, because this ligand rapidly dissociates in early endosomes regardless of the dimerization state of its receptor.

EGF-driven homodimers of ErbB-1 are degraded, but heterodimers are recycled to the cell surface

To monitor the fate of ErbB-1 after ligand-induced endocytosis, we induced down-regulation of this receptor using an unlabeled ligand and then determined the status of the remaining surface-associated binding sites by performing a radio-receptor assay. The results of this experiment revealed that ErbB-1 was destined for different fates depending on the activating ligand; upon EGF binding ErbB-1 rapidly disappeared from the surface of D1 cells, but both TGF α and E4T caused re-appearance of binding sites following an initial phase of receptor down-regulation (Figure 6). That re-appearance was due to recycling of endocytosed receptors was indicated by its complete inhibition by monensin (Figure 6, right column), a drug known to inhibit recycling of transmembrane receptors (Basu *et al.*, 1981), including the EGF-receptor (Gladhaug

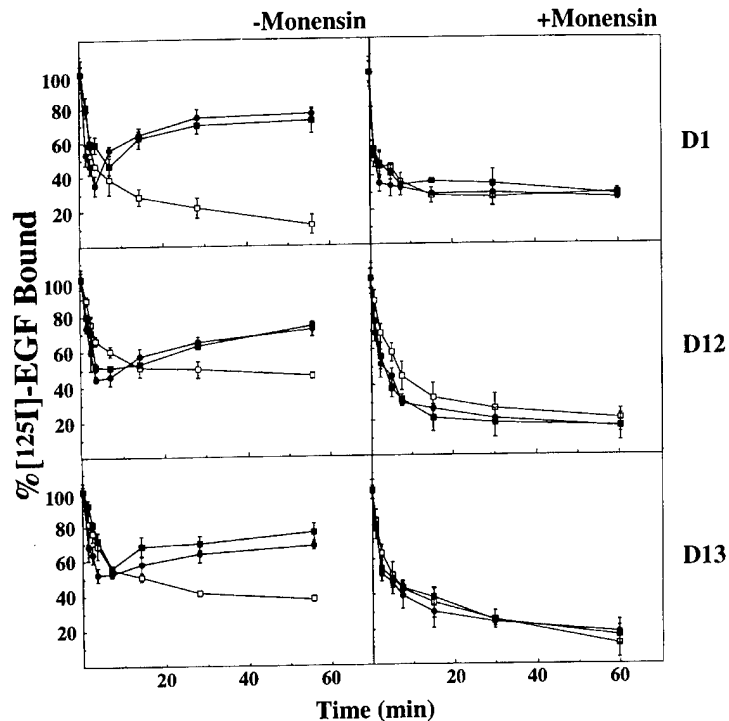


Fig. 6. Dependence of down-regulation and recycling of ErbB-1 on ligand identity and receptor interactions. The indicated derivatives of 32D cells (1.0×10^6 cells per each data point) were incubated for 2 h at 4°C with the following ligands (each at 60 ng/ml): EGF (□), TGFα (■) or E4T (●), in the absence (left panels) or presence (right panels) of monensin (0.3 mM). The cells were then transferred to 37°C and incubated for the indicated time intervals. The residual level of surface receptor that did not undergo down-regulation was determined by performing a direct binding assay with radiolabeled EGF. The results are calculated as the fraction of the initial binding of [125 I]EGF at $t=0$, and are presented as the mean \pm SD. The experiment was performed in duplicate and repeated twice.

and Christofferson, 1988). It is worthwhile noting, however, that monensin may affect other intracellular processes. For example, it has been reported that treatment with monensin can inhibit the addition of N-linked oligosaccharide chains to ErbB-1 (Mayes and Waterfield, 1984). The patterns of receptor down-regulation exhibited by EGF-treated D12 and D13 cells were different; whereas the behavior of TGFα- or E4T-driven receptors was not significantly altered by either co-receptor, in the presence of either ErbB-2 or ErbB-3 the EGF-induced down-regulation was decelerated and eventually reached a relatively high steady state (D12 and D13 panels in Figure 6). This effect was more pronounced in the case of D12 cells, in correlation with the observation that ErbB-2 potentiates EGF signaling better than does ErbB-3 (Figure 1). The relatively high steady-state of ErbB-1, that was induced by the presence of ErbB-2 or ErbB-3, was completely abolished by monensin (Figure 6). The absence of net re-appearance of binding sites, following an initial drop, in the case of EGF-treated D12 and D13 cells is attributed to the combined contribution of homodimers (that are destined for degradation) and heterodimers (that are destined for recycling). Thereby, heterodimer formation can alter the endocytic fate of an EGF-driven ErbB-1 from degradation to recycling. This scenario is consistent with the observation that the two co-receptors destabilized EGF binding at moderately acidic conditions (Figure 5), and they also attenuated both the rate of EGF uptake (Figure 3B) and the rate of ligand disappearance from the growth medium (Figure 3A).

EGF and TGFα similarly recruit ErbB-2, but engagement of ErbB-3 by heterodimerization is limited

The specificity of the potentiating effect of ErbB-2 to EGF action, but not to the biological effect of TGFα, may be explained by an alternative model which argues that TGFα less efficiently recruits ErbB-2 into heterodimers with ErbB-1 (Gulliford *et al.*, 1997), and therefore its action is unaffected by the presence of the co-receptor. Two experimental strategies were employed in order to test the validity of this model. First, the ability of TGFα to induce heterodimers was compared with that of EGF by covalent labeling of ErbB-1 with either ligand and determination of the extent of co-precipitation of the co-receptor (either ErbB-2 or ErbB-3) with ErbB-1. The results of this experiment indicated that EGF- and TGFα-labeled monomers (M) and dimers (D) of ErbB-1 underwent comparable co-immunoprecipitation by antibodies directed to ErbB-2 (Figure 7A), in agreement with recent reports (Beerli and Hynes, 1996; Riese *et al.*, 1996). The interaction between ErbB-3 and ErbB-1 was hardly detectable by this assay (D13 lanes in Figure 6B), confirming weak stability of the ErbB-1/ErbB-3 complex (Tzahar *et al.*, 1996). Thus, recruitment of a co-receptor cannot explain the differences between EGF and TGFα, because these ligands similarly engage ErbB-2 heterodimerization. This conclusion was independently supported by a second approach using monoclonal antibodies (mAbs) to ErbB-2, denoted L26 and L140, that respectively inhibit or only slightly affect heterodimer formation

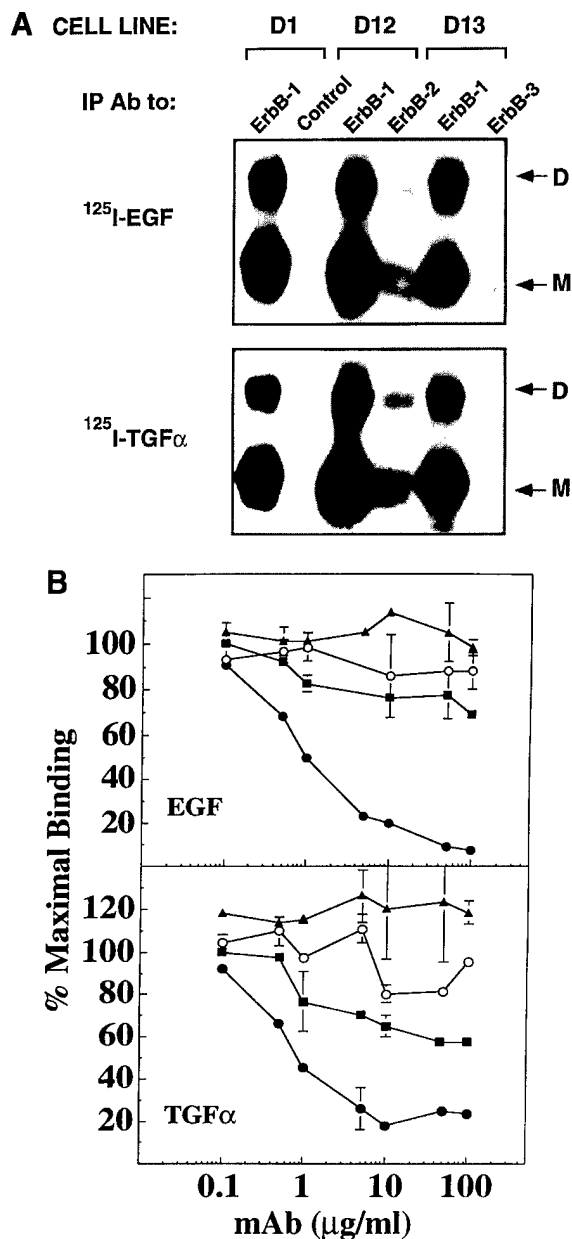


Fig. 7. Ligand-induced formation of ErbB-1-containing heterodimers. (A) D1, D12 and D13 cells were incubated with radiolabeled EGF or TGF α (each at 20 ng/ml) for 90 min at 4°C. Covalent crosslinking was performed by further incubation for 1 h with the bivalent crosslinking reagent BS³. Cell lysis and immunoprecipitation (IP) of the indicated ErbB proteins were then performed and followed by gel electrophoresis. The resulting autoradiograms are shown, along with the locations of monomeric (M_r ~180 kDa) and dimeric (D) ligand-receptor complexes. (B) D12 cells were incubated for 2 h at 4°C with either [^{125}I]EGF or [^{125}I]TGF α (each at 10 ng/ml), along with the indicated concentrations of the following anti-ErbB-2 mAbs: L26 (■) and L140 (○). For positive control we used a neutralizing antibody to ErbB-1, mAb 528 (●). As a negative control we used a mAb to a hepatitis B antigen (▲). Binding of the radiolabeled ligands was determined as described under Materials and Methods and presented as the mean \pm SD of three determinations. The experiment was repeated three times with similar results.

(Klapper *et al.*, 1997). Since by breaking ErbB-2-containing heterodimers these mAbs partly reduce the binding of ligands to their direct receptors (Klapper *et al.*, 1997), ligand binding may be used as a readout of ErbB-2 recruitment into heterodimers. When tested on D12 cells,

mAb L26 and to some extent also mAb L140 reduced binding of EGF and TGF α (Figure 7B), implying that both ligands can induce formation of the ErbB-1/ErbB-2 heterodimeric complex. Of note, in these cells TGF α was inhibited more efficiently than EGF. For control, a ligand-competitive mAb to ErbB-1 was used and it reached an almost complete inhibition of both ligands, but an irrelevant mAb was inactive (Figure 7B). Taken together, the results presented in Figure 7 exclude the possibility that differences in heterodimer recruitment account for the EGF-specific potentiating action of a co-receptor, thus strengthening an endocytosis-based mechanism of signal potentiation.

Discussion

Previous analyses concentrating on the relative mitogenic and transforming abilities of ErbB proteins and their ligands established the notion that cells co-expressing ErbB-1 together with ErbB-2 are more effectively transformed than either cells expressing ErbB-1 alone (Kokai *et al.*, 1989), or ErbB-1 in combination with ErbB-3 (Cohen *et al.*, 1996). Likewise, TGF α was shown to be more mitogenic and transforming than EGF in an autocrine or paracrine context (reviewed in Salomon *et al.*, 1995). Our present study links the superiority of receptor heterodimers with ligand specificity and provides a mechanistic basis for this functional linkage. After dealing with the proposed mechanism of signal potentiation, we discuss below the implications of our findings to current open questions, such as the extent of physiological redundancy of the multiple EGF-like ligands and the role of ErbB-2 in cancer.

The observation that ErbB-2 can *trans*-potentiate the proliferative effect of EGF more efficiently than ErbB-3 is best interpreted in terms of heterodimer formation: ErbB-1/ErbB-2 interactions are more prevalent than ErbB-1/ErbB-3 associations (Figure 7A) (Tzahar *et al.*, 1996). Nevertheless, EGF is known to activate ErbB-3 in cells overexpressing ErbB-1 (Kim *et al.*, 1994; Soltoff *et al.*, 1994), and phosphorylation of ErbB-3 apparently takes place within an EGF-driven ErbB-1/ErbB-3 heterodimer (Riese *et al.*, 1995; Pinkas-Kramarski *et al.*, 1996a; Zhang *et al.*, 1996). Thus, the relatively weak interactions between ErbB-1 and ErbB-3 may explain why the potentiating effect of ErbB-3 is weaker than that of ErbB-2 (Figure 1A). Assuming a heterodimerization model, we propose that the three ligands we tested utilize distinct mechanisms for signal potentiation. These mechanisms are described below.

EGF

According to our results, EGF can signal through two alternative pathways that are schematically presented in Figure 8. In the absence of a co-receptor, EGF is rapidly endocytosed, and due to the relatively stable binding to ErbB-1 it resists the low pH of early endosomes (Figure 5). This targets homodimeric complexes of ErbB-1, along with EGF, to degradation in lysosomes (Figure 4), and results in an almost complete disappearance of surface ErbB-1 (Figure 6). On the contrary, in the presence of a co-receptor the ternary complex (EGF, ErbB-1 and the co-receptor), whose internalization rate is relatively slow

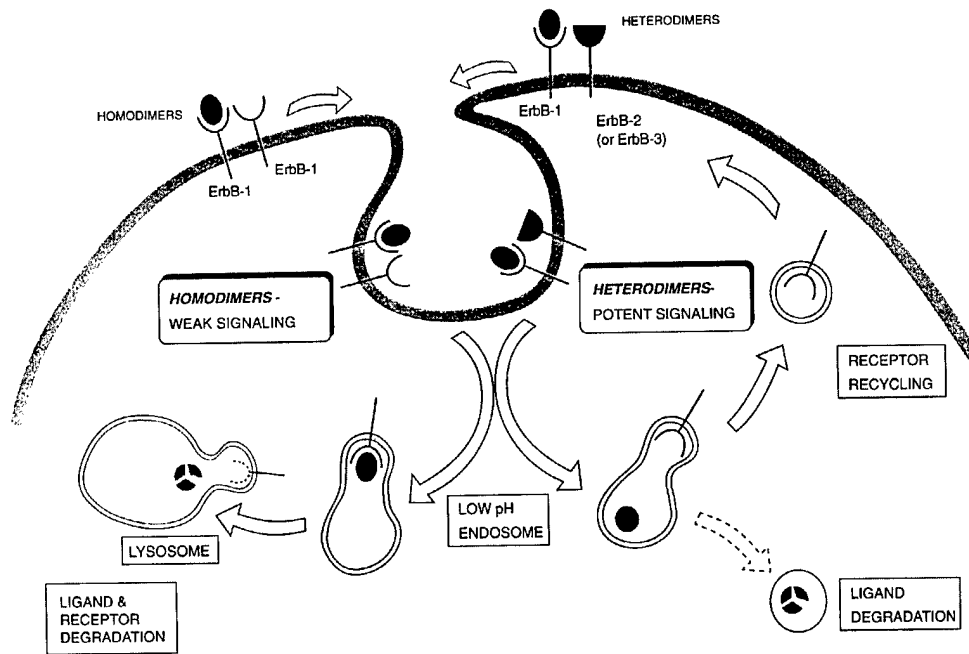


Fig. 8. Proposed endocytic model of heterodimerization-mediated tuning of mitogenic signals. EGF-occupied homodimers of ErbB-1 are destined for rapid endocytosis and lysosomal degradation that efficiently terminate signaling. In the presence of ErbB-2 (or ErbB-3), EGF signals are enhanced because ErbB-1/ErbB-2 heterodimers release EGF when the pH of early endosomes decreases. This allows recycling of the receptor back to the cell surface, thereby augmenting EGF signaling. Not presented are the pathways undertaken by TGF α and E4T. Whereas the former directs ErbB-1 to recycling regardless of the presence of a co-receptor, E4T signaling is further enhanced by its rapid on/off rates of interaction with ErbB-1. Both routes of EGF/ErbB-1 endocytosis result in intracellular degradation of the ligand, either because co-existence of homo- and hetero-dimers allows inter-pathway leakage of ligand molecules, or because the recycling route is coupled to non-lysosomal proteolytic degradation.

(Figure 3B), dissociates under the moderately acidic conditions of early endosomes (Figure 5), and consequently ErbB-1 recycles back to the cell surface (Figure 6). The exact fate of the two other molecular components of the ternary complex is unclear; whereas the co-receptor either escorts ErbB-1 to the plasma membrane, or undergoes enhanced degradation (Worthylake and Wiley, 1997), degradation of EGF takes place in an unknown compartment, probably the same non-lysosomal vesicular compartment that processes TGF α (Hamel *et al.*, 1997). Nevertheless, some recycling of undegraded EGF molecules seems to occur, as the rate of depletion of this ligand from the medium is decelerated in the presence of a co-receptor (Figure 3A). Regardless of the exact fate of their molecular components, the altered endocytic routing of ErbB-1-containing complexes may be responsible for signal potentiation, because this pathway constantly delivers unoccupied ErbB-1 molecules to the plasma membrane. By contrast, in the case of a homodimeric ErbB-1, efficient down-regulation of the receptor takes place and, therefore, signaling is short lived. It is relevant that a linkage between defective internalization of ErbB-1 and strong proliferative signals has been previously established by using an endocytosis-impaired mutant of this receptor (Wells *et al.*, 1990).

TGF α

Because binding of this ligand to both homo- and heterodimeric complexes of ErbB-1 is pH-sensitive (Figure 5), TGF α directs receptor recycling regardless of the presence of a co-receptor (Figure 6). Consequently, receptor down-regulation (Figure 6) and ligand depletion (Figure 3A) are slower in the case of TGF α than they are with EGF,

which may explain the stronger mitogenic signal of TGF α , as compared with EGF (Figure 1). In a parallel set of experiments that examined neuregulin signaling through the extremely potent ErbB-2/ErbB-3 complex we found that the cellular routing of neuregulin-driven ErbB-3 is similar to that of TGF α -driven ErbB-1 complexes (Waterman *et al.*, 1998), implying that recycling of ErbBs is a common mechanism of signal potentiation. Interestingly, however, the cellular context may affect intracellular routing of TGF α as human endometrial and other cells display more rapid processing of this ligand relative to EGF, and this correlates with biological potency (Korc and Finman, 1989; Reddy *et al.*, 1996b).

E4T

Unlike EGF and TGF α which differ only slightly in binding parameters (Figure 2), examination of the rate of dissociation of the chimeric superagonist E4T revealed a relatively high rate of release from both homo- and hetero-dimeric receptor complexes (Figure 2B). This was confirmed using plasmon resonance to measure in real time the association and dissociation rates of the three ligands from a soluble form of ErbB-1; E4T was found to behave differently to EGF and TGF α , in having both a relatively high association and dissociation rate constant (A.E.G. Lenferink and M.D.O'Connor-McCourt, manuscript in preparation). This kinetic combination may explain why the apparent affinity of E4T is similar to that of EGF or TGF α (Figure 2A). In addition, E4T displayed several significant landmarks, such as relatively slow rates of endocytosis (Figure 3B) and intracellular degradation (Figure 4), combined with pH-sensitive receptor binding (Figure 5), and an ability to induce receptor recycling

(Figure 6). It is relevant that a mutant form of EGF, denoted EGF-Val-47, shares with E4T resistance to intracellular degradation and high biological potency (Walker *et al.*, 1990). Collectively, the biochemical features of E4T appear to contribute to high signaling potency in the following way: due to its rapid on/off kinetics, E4T only transiently stimulates its receptor and therefore this ligand causes inefficient endocytosis. Moreover, due to their pH sensitivity, those E4T-bound ErbB-1 molecules that eventually undergo endocytosis rapidly recycle back to the cell surface, probably along with the chimeric ligand. Thus, the relatively strong mitogenic signal of E4T may be entirely due to inefficient signal inactivation processes. An alternative interpretation emerged from a study performed with a chimeric ligand similar to E4T (Puddicombe *et al.*, 1996). Like E4T, the other chimera displayed superagonist activity and its rate of depletion from the growth medium was relatively low. However, it has been noted that activation of receptor autophosphorylation by this ligand was more sustained than by EGF, and its mitogenic superiority displayed cell type specificity, suggesting a contextual requirement.

A central issue of the above described models of signal potentiation is the assumption that heterodimer formation by ErbB-1 can affect intracellular routing of this receptor. Most likely heterodimers do not dissociate upon endocytosis, thereby allowing an 'in trans' effect of the co-receptor on the rate and destination of receptor endocytosis. It has been shown previously that the rates of ligand internalization and receptor down-regulation are high in the case of ErbB-1 and relatively low in the case of ErbB-2, ErbB-3 and ErbB-4 (Baulida *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996a). Because ErbB-3 is practically devoid of tyrosine kinase activity (Guy *et al.*, 1994), and a kinase-defective mutant of ErbB-1 displays altered routing (Glenney *et al.*, 1988; Felder *et al.*, 1990), it is understandable why ErbB-3-containing heterodimers are less efficiently endocytosed. In fact, our recent results indicate that ErbB-3 undergoes slow endocytosis, which is followed by rapid recycling to the cell surface (Waterman *et al.*, 1998), a route that is apparently shared with a kinase-defective mutant of ErbB-1. On the other hand, the slow endocytic rates of ErbB-2 and ErbB-4 are more difficult to reason. One potential explanation may involve their inability to recruit components of the coated pit, such as the adapter protein 2 (Baulida *et al.*, 1996), which are necessary for rapid internalization. Alternatively, signals inhibitory for rapid internalization may reside in the structurally distinct cytoplasmic portions of the co-receptors (Sorkin *et al.*, 1993).

What is the physiological role of *trans*-potentiation through heterodimer formation? An evolutionary perspective may provide a hint to the answer; while only one EGF-like ligand and one ErbB-like receptor exist in worms (Kornfeld, 1997), several dozen ligands and four receptors are known in mammals. This evolutionary expansion of the number of distinct components was probably aimed at increasing physiological versatility. One such mechanism emerges from the present study: controlled expression of a co-receptor may confer superior signaling properties to others. By inference, the multiple ligands of ErbB-1 may not have redundant functions; within the appropriate context of a receptor and a co-receptor some ligands may

be superior to others. An example from mammals may demonstrate the issue: whereas normal hepatocytes respond to TGF α better than to EGF (Guren *et al.*, 1996), their embryonic counterparts respond equally well to the two ligands (Lipeski *et al.*, 1996), in accordance with the presence of ErbB-2 in fetal cells (W.E. Russell, personal communication) but not in adult hepatocytes (Carver *et al.*, 1996).

The biochemical mechanism underlying the prognostic value of ErbB-2 in human cancer is currently unclear (Hynes and Stern, 1994; Stancovski *et al.*, 1994). According to an autonomous type of mechanism, ErbB-2 contributes to high proliferation and tissue invasion perhaps because its direct ligand, whose identity is unknown, activates homodimeric ErbB-2 complexes in a manner similar to an oncogenic rat mutation (Weiner *et al.*, 1989). Alternatively, an overexpressed ErbB-2 is oncogenic perhaps because the basal tyrosine kinase activity of this receptor is relatively high (Lonardo *et al.*, 1990). The non-autonomous type of mechanism (Tzahar and Yarden, 1998) implies that ErbB-2 functions solely as a molecular amplifier of signaling initiated by all stromal EGF-like ligands (Karunakaran *et al.*, 1996), because this receptor is the preferred heterodimeric partner of all ErbB proteins (Tzahar *et al.*, 1996; Graus-Porta *et al.*, 1997), and its coupling to the MAPK pathway is extremely efficient (Ben-Levy *et al.*, 1994). The realization that ErbB-2 is a slowly internalizing receptor that can *trans*-potentiate EGF signaling by decelerating the relatively fast rate of ErbB-1 endocytosis (Figure 8) suggests that ErbB-2 supports oncogenesis not only by decelerating the rate of growth factor dissociation from heterodimeric receptor complexes (Karunakaran *et al.*, 1996), but also by delaying their inactivation process. One immediate implication is that ErbB-2 overexpression in carcinomas may be related to the type of stromal ligands expressed in the vicinity of each particular tumor. Likewise, this mechanism may be critical in metastasis; successful seeding of ErbB-2-overexpressing tumor cells at selected sites may be determined by the presence of ligands whose action is potentiated by the co-receptor. Establishment of this and other predictions made on the basis of the *trans*-potentiation effect of ErbB-2 will require additional studies.

Materials and methods

Materials, buffers and antibodies

Human recombinant EGF and TGF α were obtained from Boehringer Mannheim. Binding buffer contained RPMI-1640 medium supplemented with 0.5% bovine serum albumin (BSA). mAbs L26 and L140 raised against the extracellular part of the human ErbB-2 receptor were as described (Klapper *et al.*, 1997). mAb 528 directed against the extracellular domain of ErbB-1 was a kind gift of John Mendelsohn (MD Anderson Cancer Center, TX). The acidic ligand-strip buffer (pH 2.5) contained 5 mM acetic acid, 2.5 mM KCl, and 135 mM NaCl. Solubilization buffer contained 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM Na₃VO₄, 5 μ g/ml pepstatin A, 5 μ g/ml leupeptin and 5 μ g/ml aprotinin. HNTG buffer contained 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol.

Mutant growth factor production

The chimeric growth factor E4T, consisting of EGF sequences N-terminal to the fourth cysteine of the EGF-like motif and TGF α sequences C-terminal to this cysteine, was constructed as described (Kramer *et al.*,

1994), cloned into the pEZZ18 expression vector (Pharmacia, Uppsala, Sweden) (van de Poll *et al.*, 1995) and harvested as a secreted protein A-containing product from the periplasmic space of *Escherichia coli* KS474, a protease-deficient mutant (Strauch *et al.*, 1989). Bacteria were grown overnight in 2YTE medium under continuous agitation (200 r.p.m.). The fusion protein was isolated as described (Nilsson and Abrahmsen, 1990) and purified using IgG-Sepharose (Pharmacia). Protein yield was determined by using a binding competition assay with biotin-labeled protein A (van Zoelen *et al.*, 1993). E4T was enzymatically cleaved from protein A by factor X digestion and separated by an additional run over an IgG column. Final purification of the sample was done by reverse-phase chromatography as described previously (van de Poll *et al.*, 1995). Fractions of 1 ml were collected and tested for binding to HER-14 cells (Lenferink *et al.*, 1997). The quantity of E4T was calculated using the peak area representing the binding activity at 229 nm in the chromatography profile. Murine EGF from a natural source was used under the same experimental conditions as a standard (van de Poll *et al.*, 1995).

Cell culture

32D murine myeloid cells (Greenberger *et al.*, 1983), transfected with the various combinations of erbB-encoding plasmid or viral vectors (Pinkas-Kramarski *et al.*, 1996a) were grown in RPMI-1640 medium supplemented with antibiotics, 10% heat-inactivated fetal bovine serum and 0.1% medium conditioned by IL-3-producing X63/0 cells (Karasuyama and Melchers, 1988). Cells were kept under continuous selection using 0.4 mg/ml hygromycin B (Boehringer Mannheim) for D1 cells and additionally 0.6 mg/ml G418 (Boehringer Mannheim) for D12 and D13 cells. NIH 3T3 cells transfected with the wild-type human EGF receptor (HER-14 cells) and expressing 4.0×10^5 ErbB-1 molecules/cell (Honegger *et al.*, 1988), were cultured in gelatinized flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum.

Ligand displacement assays

The rationale of this assay was adopted and modified from a previous protocol (Reddy *et al.*, 1996a). Essentially, ligand concentration profiles were determined in media conditioned by preincubation with ErbB-expressing cells. Because a radioimmunoassay and a radio-receptor assay were less satisfactory, we used a bioassay with HER-14 murine fibroblasts overexpressing ErbB-1. Recombinant human EGF, TGF α and the chimera E4T were radiolabeled using the indirect iodogen method (Pierce, Roxford, IL), as described previously (Peles *et al.*, 1993). For ligand displacement analysis, 1.0×10^6 cells were washed once with binding buffer, incubated with a radiolabeled ligand (at 1 ng/ml) for 2 h at 4°C in 0.2 ml of the same buffer, containing serial dilutions of the unlabeled ligand. To terminate ligand binding, cells were sedimented (9000 g, 2 min), washed once with 0.5 ml binding buffer and loaded on top of a 0.7 ml cushion of BSA. Tubes were spun again to remove the unbound ligand and radioactivity in the cell pellets was counted directly.

Cellular proliferation assays

To analyze ligand-induced proliferative responses of D1, D12 and D13 cells, 5.0×10^4 cells were washed free of IL-3, resuspended in RPMI-1640 and seeded in 96-wells plates. For dose-response experiments, serial dilutions of a ligand were added in RPMI-1640 medium and cells were incubated for 24 h at 37°C. IL-3 (1:1000 of medium conditioned by a producer cell line) was used as a positive control. Proliferation was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which determines mitochondrial activity in living cells (Mosman, 1983). During an incubation for 2 h at 37°C with MTT, living cells transform the tetrazolium ring into dark blue formazan crystals which can be quantified by reading the optical density at 540–630 nm after lysis of the cells with acidic 2-propanol. For cell survival experiments, cells were seeded at the same density in 96-well plates and incubated at 37°C with a fixed ligand concentration (100 ng/ml). Cell survival was determined 24, 48 and 72 h after ligand addition using the MTT method.

Ligand depletion assay

The rationale of this assay was adopted and modified from a previous protocol (Reddy *et al.*, 1996a). Essentially, ligand concentration profiles were determined in media conditioned by preincubation with ErbB-expressing 32D cells. Because radioimmunoassay and radio-receptor assay was less satisfactory we used a bioassay with HER-14 fibroblasts overexpressing ErbB-1. HER-14 cells were seeded in gelatinized 24-well dishes (1.8 cm²) at a density of 6.0×10^4 cells/well in 1 ml DMEM/

10% serum. After 24 h of incubation the medium was replaced by 0.9 ml of DMEM/Ham's F12 medium (1:1) supplemented with 30 nM Na₂SeO₃, 10 µg/ml human transferrin and 0.5% BSA. After an additional incubation for 48 h, 0.1 ml medium that was conditioned for 24 h by D1, D12 or D13 cells was added. Eight hours later 0.5 µCi [³H]thymidine (TdR) was added in 0.1 ml Ham's F12 medium. Incorporation of the tracer into cellular DNA was determined 24 h after growth factor addition as described previously (van Zoelen *et al.*, 1986).

Receptor recycling assays

To quantify receptor recycling, 1.0×10^6 cells were incubated for 2 h at 4°C with various ligands (at 60 ng/ml) in the absence or presence of 0.3 mM monensin (as indicated), and then transferred to 37°C for various time periods. Subsequently, cells were sedimented (9000 g, 2 min), resuspended, and incubated in ice-cold ligand-strip buffer for 2 min on ice. Cells were sedimented again, neutralized in binding buffer and incubated in the same buffer for an additional 1 h at 37°C to allow intact internalized receptors to recycle to the cell surface. To quantify the number of ErbB-1 molecules on the cell surface, cells were incubated for 2 h at 4°C with [¹²⁵I]EGF, sedimented as above, rinsed once in binding buffer and spun through a serum cushion to remove the unbound ligand, prior to γ -counting.

Ligand internalization assays

The fate of various ligands was determined by incubating 32D cells (1.0×10^6 cells) with 1 ng/ml radiolabeled EGF, TGF α or E4T. Following 2 h at 4°C cells were washed in binding buffer, resuspended in the same buffer that contained unlabeled ligand (3 µg/ml) and transferred to 37°C for the indicated time periods. Then, cells were immediately cooled on ice, incubated for 5 min in the acidic ligand-strip buffer (pH 2.5), and sedimented through a serum cushion. The released ligand was considered as cell surface-associated ligand. Cells were lysed in 1% Triton X-100 for 1 h at room temperature prior to γ -counting.

Ligand dissociation assays

Dissociation of radiolabeled human EGF, TGF α and E4T was investigated using 1.0×10^6 D1, D12 or D13 cells. Cells were rinsed once in binding buffer and subsequently incubated (2 h, 4°C) with excess (60 ng/ml) radiolabeled ligand in binding buffer. Then, the tubes were spun and the cell pellet was resuspended and incubated at 4°C in binding buffer supplemented with 3 µg/ml unlabeled ligand for the indicated time spans. Finally, cells were pelleted and lysed in 100 mM NaOH containing 0.1% sodium dodecylsulfate prior to γ -counting.

Ligand degradation assays

Derivatives of 32D cells (1.0×10^6 cells) were washed free of IL-3 and subsequently incubated at 20°C for 60 min with radiolabeled ligand (at 1 nM) in binding buffer. Then, cells were spun through a serum cushion to remove the unbound ligand and incubated, without ligand, for up to 240 min at 37°C. At various time points, trichloroacetic acid-precipitable counts in the medium (degraded ligand) were determined.

Ligand crosslinking analyses

For chemical crosslinking experiments with 32D cells, 5.0×10^6 cells were incubated for 2 h on ice with 20 ng/ml radiolabeled EGF or TGF α . The chemical crosslinker bis(sulfonylsuccinimide)-suberate (BS³, Pierce, Roxford, IL) was added to a final concentration of 1 mM. Cells were then incubated for 45 min at 4°C and subsequently washed with phosphate buffered saline, pelleted by centrifugation, and lysed in solubilization buffer. Lysates were cleared by centrifugation, and immunoprecipitated with antibodies against specific ErbB proteins. Rabbit antibodies were directly coupled to protein A-Sepharose beads while shaking (1 h, 4°C); mouse antibodies were coupled indirectly using rabbit-anti-mouse IgG under the same conditions. ErbB proteins present in the cell lysate were immunoprecipitated with the protein A-Sepharose-antibody complex for 2 h at 4°C. Precipitates were washed three times in HNTG buffer prior to heating for 5 min at 95°C in gel sample buffer under reducing conditions. Samples were analyzed using gel electrophoresis (7.5% acrylamide).

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Epiregulin Is a Potent Pan-ErbB Ligand That Preferentially Activates Heterodimeric Receptor Complexes*

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The ErbB signaling network consists of four transmembrane receptor tyrosine kinases and more than a dozen ligands sharing an epidermal growth factor (EGF) motif. The multiplicity of ErbB-specific ligands is incompletely understood in terms of signal specificity because all ErbB molecules signal through partially overlapping pathways. Here we addressed the action of epiregulin, a recently isolated ligand of ErbB-1. By employing a set of factor-dependent cell lines engineered to express individual ErbBs or their combinations, we found that epiregulin is the broadest specificity EGF-like ligand so far characterized: not only does it stimulate homodimers of both ErbB-1 and ErbB-4, it also activates all possible heterodimeric ErbB complexes. Consistent with its relaxed selectivity, epiregulin binds the various receptor combinations with an affinity that is approximately 100-fold lower than the affinity of ligands with more stringent selectivity, including EGF. Nevertheless, epiregulin's action upon most receptor combinations transmits a more potent mitogenic signal than does EGF. This remarkable discrepancy between binding affinity and bioactivity is permitted by a mechanism that prevents receptor down-regulation, and results in a weak, but prolonged, state of receptor activation.

Various biological processes are controlled by intercellular interactions that are mediated by polypeptide growth factors. Examples include embryonic development, neuronal functions, hematopoiesis, and pathological situations, like wound healing and malignant transformation. The mechanism transmitting extracellular signals ultimately starts with binding of the growth factor to a cell surface receptor, that in many cases carries an intrinsic tyrosine kinase activity (1). These receptors fall into several subgroups sharing structural and functional characteristics. Each subgroup of receptors specifically recognizes a family of structurally homologous growth factors. Perhaps the most striking multiplicity of related growth factors is exemplified by the epidermal growth factor (EGF)¹ family of molecules (2). This six cysteine-containing motif of 45–60 amino

acids is shared by all members of the family, and it functions as the receptor binding portion of the molecule. Currently there are four known receptors for EGF-like ligands, constituting the ErbB subgroup of receptor tyrosine kinases (also known as HER, or type I receptor tyrosine kinases (3)). Whereas ErbB-1 binds many ligands, including EGF, transforming growth factor α (TGF α), and amphiregulin, both ErbB-3 and ErbB-4 bind to a family of isoforms, collectively known as neuregulins (also called Neu differentiation factors, heregulins, glial growth factors, and acetylcholine receptor inducing activity) (4). A related group of molecules, termed NRG2, binds to the same two receptors (5–7), and a third molecule, NRG3, exclusively binds to ErbB-4 (8). Two other ligands, betacellulin (9), and the heparin-binding EGF-like growth factor (10, 11) bind to both ErbB-1 and ErbB-4. Interestingly, the most oncogenic member of the ErbB family, ErbB-2, binds none of the EGF-like ligands with high affinity. However, recent studies indicate that ErbB-2 functions as a shared low affinity receptor that binds the apparently bivalent EGF-like ligands with low affinity, once they are presented by either one of the high affinity receptors (12).

Despite shared receptor specificity, it is clear that the multiple EGF-like ligands play distinct physiological roles: gene targeting experiments showed that loss of function of ErbB-1 (13–15) more severely impairs embryonic development than inactivation of one of its ligands, TGF α (16). On the other hand, targeting of either neuregulin (17), ErbB-2 (18), or ErbB-4 (19), resulted in the same embryonic cardiac defect, indicating that activation of an ErbB-2/ErbB-4 receptor combination is exclusively mediated by neuregulin in the developing heart. That ligand multiplicity related to tissue-specific expression is suggested by distinct spatial and temporal patterns of expression of the various ligands (reviewed in Ref. 2), and also by experiments with transgenic mice demonstrating tissue selectivity of specific ErbB-1 ligands (20). Part of the physiological selectivity of ligands with shared receptors may be attributed to their domains that flank the EGF-like motif, including the presence of heparin-binding sites, sugars, and specific protein motifs.

In this study we addressed the functional identity of epiregulin, a recently identified ligand of ErbB-1 (21, 22). Like TGF α , this ligand was originally isolated from the medium of transformed fibroblasts, and its transmembrane precursor carries only short sequences that flank the EGF-like motif. Epiregulin expression is relatively restricted; except for macrophages and placenta, other human tissues contain very low or no epiregulin transcripts, but most types of epithelial tumors are characterized by high expression of the growth factor (23). Although

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¹ The abbreviations used are: EGF, epidermal growth factor; CHO, Chinese hamster ovary; IL-3, interleukin 3; mAb, monoclonal antibody;

MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NDF, Neu differentiation factor; TGF- α , transforming growth factor α .

epiregulin competed with EGF on the binding to ErbB-1, it displayed relatively low affinity to ErbB-1-overexpressing cells (21). On the other hand, the factor displayed dual biological function *in vitro*: it stimulated proliferation of fibroblasts, smooth muscle cells, and hepatocytes, but inhibited growth of several tumor-derived epithelial cell lines (21). These observations, and the emerging broader than expected specificity of EGF-like ligands to ErbB proteins (reviewed in Ref. 24), prompted us to analyze the selectivity of epiregulin to ErbB proteins. Here we report that epiregulin is a pan-ErbB ligand that activates all ligand-stimulatable combinations of ErbB proteins with variable efficiency. Strikingly, in a model cellular system, epiregulin more potently activates mitogenesis than does EGF, although the affinity of EGF to ErbB-1 is approximately 100-fold higher. This superiority of epiregulin is independent on the presence of other ErbB proteins, and appears to result from a relatively inefficient mechanism of receptor inactivation.

EXPERIMENTAL PROCEDURES

Materials, Buffers, and Antibodies—A recombinant form of NDF- $\beta_{177-246}$ was kindly provided by Amgen (Thousand Oaks, CA). Human recombinant EGF and TGF α were purchased from Sigma. Radioactive materials were purchased from Amersham (Buckinghamshire, United Kingdom). IODO-GEN and BS³ were from Pierce. A monoclonal antibody to the ErbB-2 protein, mAb L26 (25), was used to stimulate ErbB-2. A monoclonal anti-phosphotyrosine antibody (PY-20, Santa Cruz Biotechnology) was used for Western blot analysis. A mAb to the active form of MAPK (doubly phosphorylated on both tyrosine and threonine residues of the TEY motif) (26) was a gift from Rony Seger. Binding buffer contained Dulbecco's modified Eagle's medium with 0.5% bovine serum albumin and 20 mM HEPES. Solubilization buffer contained 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM EGTA, 1.5 mM MgCl₂, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 trypsin inhibitor unit/ml), and 10 μ g/ml leupeptin.

Peptide Synthesis—Epiregulin was synthesized on an Applied Biosystems (ABI) model 431 peptide synthesizer fortified with UV feedback monitoring at 301 nm, and using Fmoc (9-fluorenylthoxycarbonyl)-Rink amide AM resin. Only the EGF-like domain of the murine epiregulin (21) was synthesized. The conventional ABI monitor previous peak algorithm was employed up to five times with a cut-off of 3.5% of the first deprotection. A secondary deprotection was performed and followed by double coupling. Acetic anhydride/1-hydroxybenzotriazole capping was utilized at the end of each coupling, followed by washing with 1:1 trifluoroethanol/dichloromethane. The peptide was deprotected and removed from the resin as described (27), with the following modifications: methoxyindole (2%) was added to reagent K, and the reaction time was changed to 3.5 h. Small quantities of the reduced peptides were purified by reverse-phase high performance liquid chromatography and examined by matrix-assisted laser desorption ionization mass spectral analysis. The crude reduced protein was dissolved in a Tris-HCl buffer, pH 6.0, containing guanidium HCl (6 M) and diluted to a concentration of 0.06 mg/ml in methionine-containing buffer (10 mM) that included 1.5 mM cystine, 0.75 mM cysteine, and 100 mM Tris, pH 8.0. The mixture was stirred for 48 h at 4 °C, and the oxidized protein isolated on a C-4 VYDAC 10 micron preparative column (22 \times 250 mm) using a 0.1% trifluoroacetic acid/water/acetonitrile gradient. The oxidized protein was lyophilized and characterized by mass spectrometry and amino acid analysis, and shown to be homogeneous. Electrospray mass spectrometry was used to verify the mass of the synthetic peptide.

Cell Lines—MDA-MB453 cells were purchased from the American Type Culture Collection (Rockville, MD). The Chinese hamster ovary (CHO) cell lines expressing various ErbB proteins or their combinations were described previously (28). The establishment of a series of interleukin 3 (IL-3)-dependent 32D myeloid cells expressing all combinations of ErbB-1, ErbB-2, and ErbB-3 has been described (29). To generate an ErbB-4-overexpressing derivative of 32D cells, we used an LTR-*erbB-4* expression vector that was electroporated into 32D cells as described (30). Cell lines co-expressing ErbB-2 or ErbB-3, together with ErbB-4, were established by transfection of the pLXSHD retroviral vector (31), directing ErbB-4 expression, into ErbB-2- or ErbB-3-expressing cells (D2 and D3, respectively) by using electroporation (BioRad GenePulser, set at 400 volts and 250 millifarad). After a 24-h long

recovery, cells were selected for 4–5 weeks in medium containing histidinol (0.4 mg/ml). Clones expressing the two receptors were identified by using Western blotting, and isolated by limiting dilution. Due to differences in promoter potency, the selected cell line that singly expresses ErbB-4 (E4 cells) contained approximately 10–12-fold more ErbB-4 molecules than cell lines expressing the combinations of ErbB-4 with ErbB-2 (D24 cells) or with ErbB-3 (D34 cells). A cell line expressing only approximately 5×10^4 ErbB-4 molecules per cell was established by using previously described procedures (29) and denoted D4.

Radiolabeling of Ligands, Covalent Cross-linking, and Ligand Binding Analyses—Growth factors were labeled by using IODO-GEN as described (32). The specific activity was approximately 5×10^6 cpm/ng. For covalent cross-linking analysis, cells (10^6) were incubated on ice for 1.5 h with ¹²⁵I-EGF, ¹²⁵I-NDF- β_1 , or ¹²⁵I-epiregulin (each at 100 ng/ml). The chemical cross-linking reagent BS³ was then added (1 mM), and after 90 min on ice, cells were pelleted and solubilized in solubilization buffer. For analyses of ligand displacement with 32D cells, 10^6 cells were washed once with binding buffer, and then incubated for 2 h at 4 °C with a radiolabeled ligand (1 ng/ml) and various concentrations of an unlabeled ligand in a final volume of 0.2 ml. Nonspecific binding was determined in the presence of a 100-fold molar excess of the unlabeled ligand. To terminate ligand binding, each reaction tube was washed once with 0.5 ml of binding buffer and loaded on top of a 0.7-ml cushion of bovine serum. The tubes were spun (12,000 $\times g$, 2 min) to remove the unbound ligand. Ligand displacement from CHO cells was analyzed with cell monolayers grown in 24-well dishes. Monolayers were washed once with binding buffer and then incubated for 2 h at 4 °C with 1 ng/ml of the radiolabeled ligand, along with increasing concentrations of an unlabeled growth factor. Then, cells were washed three times with ice-cold binding buffer. Labeled cells were lysed for 15 min at 37 °C in 0.5 ml of 0.1 N NaOH solution containing 0.1% sodium dodecyl sulfate, and the radioactivity was determined. Nonspecific binding was calculated by subtracting the binding of radiolabeled ligands to untransfected CHO cells, or by performing the binding assays in the presence of a 100-fold excess of the unlabeled ligand.

Receptor Down-regulation Assay—Ligand-induced receptor down-regulation was measured as follows: cells grown in 24-well plates were incubated at 37 °C for up to 90 min without or with various ligands in binding buffer. The cells were then put on ice, rinsed twice with binding buffer, and surface-bound ligand molecules removed by using a 7-min long incubation in 0.5 ml of solution of 150 mM acetic acid, pH 2.7, containing 150 mM NaCl (33). The number of ligand-binding sites that remained exposed on the cell surface was then determined by incubating cells at 4 °C with radiolabeled EGF (20 ng/ml) for 90 min.

Lysate Preparation and Western Blotting—For analysis of total cell lysates, gel sample buffer was added directly to cell monolayers or suspensions. For other experiments, solubilization buffer was added to cells on ice. The adherent CHO cells were scraped with a rubber policeman into 1 ml of buffer, transferred to microtubes, mixed harshly, and centrifuged (10,000 $\times g$, 10 min at 4 °C). Samples were resolved by gel electrophoresis through 7.5% acrylamide gels, and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 2 h in TBST buffer (0.02 Tris-HCl buffered at pH 7.5, 0.15 M NaCl, and 0.05% Tween 20) containing 1% milk, blotted for 2 h with 1 μ g/ml primary antibody, washed, and reblotted with 0.5 μ g/ml secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected with an enhanced chemiluminescence reagent (Amersham Corp.).

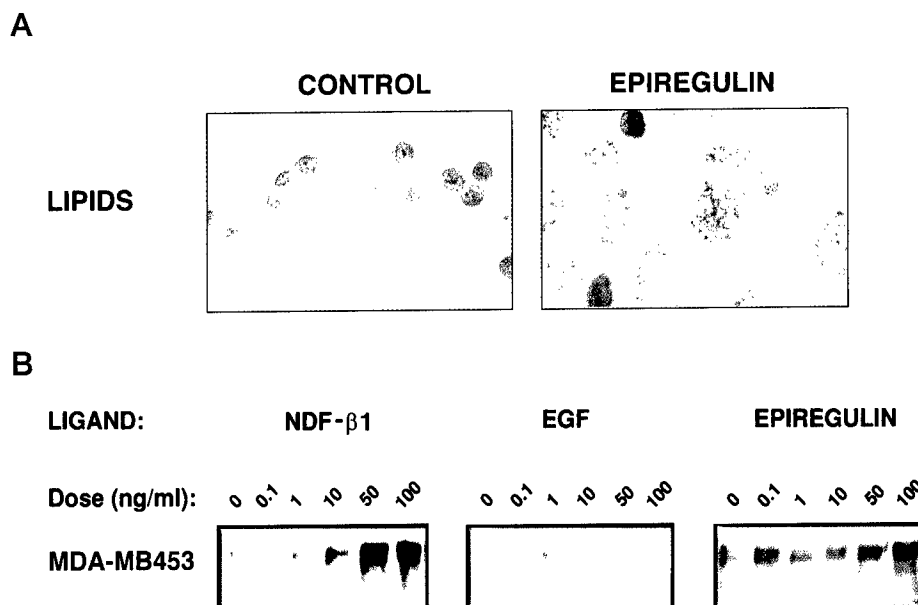
Cell Proliferation Assays—Cells were washed free of IL-3, resuspended in RPMI 1640 medium at 5×10^5 cells/ml, and treated without or with growth factors or IL-3 (1:1000 dilution of medium conditioned by IL-3-producing cells). Cell survival was determined by using the MTT assay as described previously (29). MTT (0.1 mg/ml) was incubated for 2 h at 37 °C with the analyzed cells. Living cells can transform the tetrazolium ring into dark blue formazan crystals, that can be quantified by reading the optical density at 540–630 nm after lysis of the cells with acidic isopropyl alcohol (34).

Cellular Differentiation Assays—MDA-MB453 human mammary cancer cells were plated in chamber slides (Lab-Tek) and then incubated for 4 days in the absence or presence of ligands (50 ng/ml). Cells were stained with oil red O, to visualize neutral lipids, as described previously (35).

RESULTS

Induction of Cellular Differentiation and Tyrosine Phosphorylation by Epiregulin in the Absence of ErbB-1—The duality of epiregulin's activity, namely, mitogenicity for some normal

FIG. 1. Induction of cellular differentiation and tyrosine phosphorylation by epiregulin in mammary cells lacking ErbB-1/EGF receptor. A, MDA-MB453 human mammary cancer cells, that express no ErbB-1, were plated in chamber slides and then incubated for 4 days in the absence (*CONTROL*) or presence of epiregulin (50 ng/ml). Cells were stained with oil red O, to visualize neutral lipids. Note the appearance of lipid droplets (yellow) in epiregulin-treated cells. The magnification used was $\times 600$. B, following an overnight starvation, 10^6 MDA-MB453 cells were incubated for 2 min at 37°C without or with EGF, NDF- $\beta 1$, or epiregulin, at the indicated concentrations. Whole cell lysates were then prepared, resolved by gel electrophoresis, and immunoblotted with an antibody to phosphotyrosine (PY20). Bound antibody was detected by using a chemiluminescence-based method.



cells and growth inhibition of epithelial tumor cells (21), may depend on expression patterns of ErbB proteins, and thus may be explained by epiregulin's interaction with receptor species other than ErbB-1. As an initial test of this paradigm we examined the biological effect of epiregulin on MDA-MB453 mammary tumor cells, which are devoid of the EGF-receptor (ErbB-1), but can undergo phenotypic differentiation in response to EGF-like ligands (36). Evidently, these cells underwent growth arrest in response to long-term incubation with epiregulin, and displayed phenotypic differentiation that included cell flattening, and appearance of neutral lipid-containing vesicles (Fig. 1A). EGF, at 1–200 ng/ml, was inactive in inducing cell differentiation (data not shown), whereas similar phenotypic alterations were induced also by NDF/neuregulin, a ligand that interacts with both ErbB-3 and ErbB-4 (37). Consistent with their biological effects on MDA-MB453 cells, both epiregulin and NDF, but not EGF, were able to stimulate tyrosine phosphorylation of a 180-kDa protein at concentrations higher than 10 ng/ml (Fig. 1B). In conclusion, epiregulin action on the mammary epithelial cell line we examined is independent of ErbB-1, and is distinct from the effect of EGF.

Epiregulin Is a Relatively Potent Stimulator of ErbB-1, but It Can Transmit Biological Signals Also through Combinations of Other Receptors—To directly address the specificity of epiregulin to ErbB receptors, we employed a previously described series of cell lines derived from the IL-3-dependent 32D myeloid cell line (29). Parental 32D cells express no ErbB protein, but as a result of transfection, the derivative lines singly express ErbB-1, ErbB-2, ErbB-3, or ErbB-4 (cell lines denoted D1, D2, D3, and E4, respectively). Likewise, co-expression of two ErbB proteins established cell lines with various combinations. For example, D13 cells co-express ErbB-1 and ErbB-3. Analysis of cell proliferation in the absence of IL-3, but in the presence of increasing concentrations of epiregulin, EGF, or NDF- $\beta 1$, revealed several interesting characteristics of epiregulin. First, the factor was more potent than EGF on cells singly expressing ErbB-1 (D1 cells, Fig. 2A), as well as on cells expressing combinations of ErbB-1 with either ErbB-2 (D12 cells) or ErbB-3 (D13 panel in Fig. 2A). Not only were the dose-response curves of epiregulin shifted to the left, but this ligand exerted in D1 and D13 cells a higher maximal response than EGF. Consistent with the catalytic inactivity of ErbB-3 (38), and the inability of ErbB-2 to bind any of the ErbB ligands with high affinity (12), cells singly expressing ErbB-3 or ErbB-2 (D3 and D2 cell lines,

respectively) did not respond to epiregulin (Fig. 2A). For control, we verified that D2 cells are stimutable by a mAb to ErbB-2 (25) (Fig. 2A), and D3 cells retained response to IL-3 (Fig. 3). Surprisingly, E4 cells that highly overexpress ErbB-4 exhibited mitogenic response to both epiregulin and EGF at concentrations above 5 ng/ml (Fig. 2A). In fact, the response to EGF was reproducibly slightly higher than the mitogenic effect of epiregulin on these cells. Due to the use of different promoters, ErbB-4 expression in the E4 cell line was more than 10-fold higher than that of ErbB-1 in D1 cells (see "Experimental Procedures"). To address the possibility that epiregulin and EGF act through ErbB-4 only when this receptor is overexpressed, we analyzed a second cell line, D4, whose ErbB-4 expression is comparable with the level of ErbB-1 expression in D1 cells. When tested on D4 cells, both ligands displayed mitogenic activity (Fig. 2B), along with an ability to stimulate tyrosine phosphorylation (Fig. 2C). Nevertheless, in terms of the maximal response to IL-3, both epiregulin and EGF were more active on the ErbB-4-overexpressing cell line than on the low expressor D4 cells, implying that the level of expression of ErbB-4 affects the level of cell proliferation, but not ligand specificity.

Although the effect of epiregulin on cells coexpressing ErbB-3 with ErbB-1 (D13 cells) was higher than that of EGF, the response to NDF was much higher, presumably because NDF better recruits ErbB-3 into heterodimers (29, 39). Nevertheless, it is clear that also epiregulin can recruit ErbB-3 into heterodimers, as reflected by its activity on cells coexpressing a combination of ErbB-3 with either ErbB-2 (D23 cells, Fig. 2A) or ErbB-4 (D34 cells, Fig. 2A). This ability of epiregulin distinguishes it from EGF, whose signaling through the ErbB-2/ErbB-3 heterodimer occurs only at extremely high concentrations (Fig. 2A and Ref. 40 and 41), and is completely inactive in stimulating an ErbB-3/ErbB-4 heterodimer (Fig. 2A). Moreover, although EGF is slightly more potent than epiregulin on ErbB-4-expressing cells (*E4 panels* in Figs. 2A and 3), epiregulin is superior when ErbB-2 is coexpressed with ErbB-4 (*D24 panels* in Figs. 2A and 3), suggesting that this ligand is a better stimulator of the ErbB-2/ErbB-4 heterodimer. Taken together, the results presented in Fig. 2 imply that relative to EGF, epiregulin is a better agonist of ErbB-1-containing homo- and heterodimers. In addition, recruitment of ErbB-2, ErbB-3, and ErbB-4 into heterodimers is more efficient in the case of epiregulin. However, homodimers of ErbB-4 are better activated

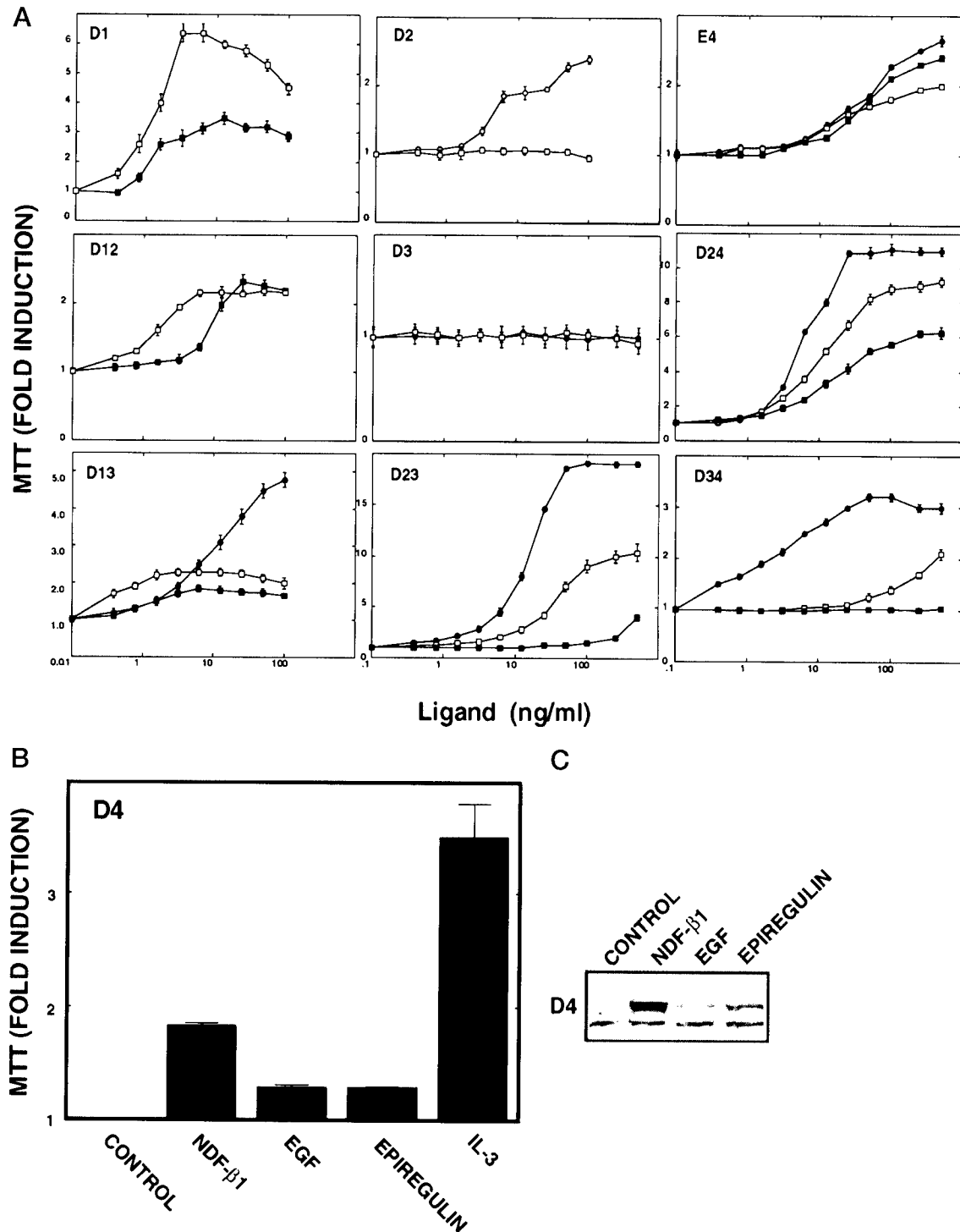


FIG. 2. Proliferative responses of ErbB-expressing derivatives of 32D myeloid cells to epiregulin and other ligands. A, the indicated sublines of 32D cells were tested for cell proliferation by using the MTT assay. D1, D2, D3, and E4 cells express ErbB-1, ErbB-2, ErbB-3, and ErbB-4, respectively, whereas the other cell lines co-express the corresponding two ErbB proteins. Cells were deprived of IL-3 and plated at a density of 5×10^5 cells/ml in media containing serial dilutions of EGF (closed squares), epiregulin (open squares), NDF- β 1 (closed circles), or a monoclonal antibody to ErbB-2 (mAb L26, open circles). The MTT assay was performed 24 h later. Results are presented as fold induction over the control untreated cells, and are the mean \pm S.D. of four determinations. Each experiment was repeated at least twice. Cells singly expressing ErbB-3 (D3 cells) responded to none of the ligands we tested, but these cells retained response to IL-3. B, D4 cells were tested for cell proliferation by using the MTT assay as described above, except that the indicated ligands were used at 100 ng/ml. For control, cells were incubated in the absence of IL-3 or ligands. C, ligand-induced tyrosine phosphorylation was analyzed in D4 cells by incubating 10^6 cells without or with the indicated ligands (each at 100 ng/ml). Following 2 min at 37 $^{\circ}$ C, whole cell lysates were prepared and analyzed by immunoblotting with a mAb to phosphotyrosine. Antibody detection was performed with a chemiluminescence kit. Only the 180-kDa region of the blot is shown.

by EGF, and neither homodimers of ErbB-2 nor ErbB-3-ErbB-3 complexes are stimutable by the two ligands.

These conclusions were further supported by long-term sur-

vival experiments that are presented in Fig. 3. In this type of analysis cells are maintained in the absence of IL-3, but in the presence of epiregulin (or other ligands) for several days, and

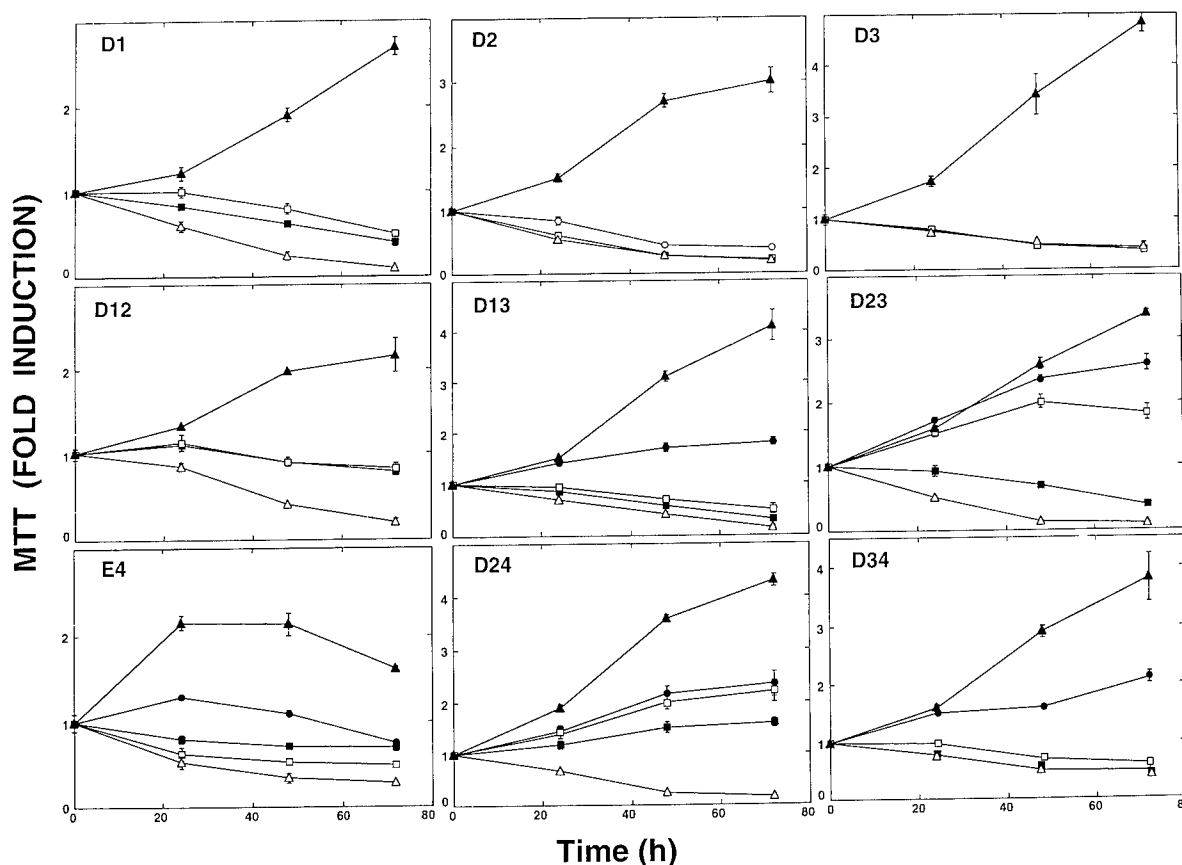


FIG. 3. Ligand-dependent survival of ErbB-expressing 32D cells in the absence of IL-3. The indicated sublines of 32D cells were incubated for various time intervals at a density of 5×10^5 cells/ml in the presence (closed triangles) or absence of IL-3 (open triangles), or with one of the following ligands, each at a concentration of 100 ng/ml (except for D23 cells, that were treated with EGF at 500 ng/ml to reflect the residual activity of this ligand through the ErbB-2/ErbB-3 heterodimer (40, 41)): EGF (closed triangles), epiregulin (open squares), NDF- β 1 (closed circles), and an antibody to ErbB-2 (mAb L26, open circles). Cell survival was determined daily by using the colorimetric MTT assay. The data presented are the mean \pm S.D. of six determinations. Each experiment was repeated at least twice.

their survival determined by using the MTT assay. Consistent with the dose curves of the short-term mitogenic assay, at a saturating concentration epiregulin acted as a slightly better survival factor than EGF for cells expressing ErbB-1, either alone or in combination with ErbB-3 (Fig. 3). Also consistent with the data of Fig. 2 was the observation that EGF exerted a better survival activity on ErbB-4-overexpressing cells (E4 panel in Fig. 3). Interestingly, the presence of ErbB-2, together with either ErbB-4 or ErbB-3, enabled epiregulin to become a potent stimulator of cell proliferation, whereas EGF acted primarily as a survival factor under these circumstances (D23 and D24 panels in Fig. 3). Although survival of cells coexpressing ErbB-3 and ErbB-4 was only slightly extended by epiregulin (D34 panel in Fig. 3), this effect was higher than that of EGF, reinforcing the relative preference of epiregulin for heterodimeric receptor combinations.

Receptor Phosphorylation and MAP Kinase Activation by Epiregulin—Signaling by all EGF-like ligands is mediated by rapid tyrosine phosphorylation of the respective receptors, and is ultimately funneled to the mitogen-activated protein kinase (MAP-kinase/Erk) pathway (42). The biological differences we observed between epiregulin, EGF, and NDF in subsets of 32D cells suggested that these ligands may differ in signaling potency, and especially in their ability to recruit the MAPK pathway. To analyze receptor phosphorylation and MAPK activation we probed blots of whole extracts, prepared from ligand-stimulated cells, with antibodies to phosphotyrosine, or with a murine mAb that specifically recognizes the active, doubly phosphorylated form of the ERK1 and ERK2 MAPKs (26). Surprisingly, the more mitogenic ligand of ErbB-1, epiregulin,

exhibited weaker, but not less sustained, tyrosine phosphorylation of proteins at the 180-kDa range corresponding to ErbB-1 in D1 cells (Fig. 4A). Although both EGF and epiregulin stimulated MAPK phosphorylation in these cells, the patterns of activation differed: a comparable increase in the activity of both forms of the kinase was induced by epiregulin, whereas primarily the lower form was activated after stimulation with EGF. Importantly, although stimulation by EGF was more uniform at intermediate time intervals (10–20 min), it completely disappeared after 30–60 min, at which time the effect of epiregulin was still detectable. By contrast, ErbB-4 phosphorylation was stronger with epiregulin than with EGF (E4 panel in Fig. 4A), although the latter is a slightly more efficient mitogen for the ErbB-4-overexpressing E4 cells (Figs. 2A and 3). These differences between ErbB-1 and ErbB-4 phosphorylation are cell-type independent, because they were reproduced in a series of CHO cells expressing ErbB-1 (CB1 cells) or ErbB-4 (CB4 cells), on a low background of the endogenous hamster ErbB-2 (Fig. 4B). Analysis of 32D cells expressing a combination of ErbB-2 with ErbB-3 (D23 cells) revealed that both forms of MAPK were rapidly stimulated by epiregulin, but phosphorylation of both ErbBs and MAPKs by EGF occurred only at very high ligand concentrations, in agreement with recent reports (40, 41). The maximal activation of MAPK in these cells was observed upon stimulation with NDF, a ligand whose mitogenic effect was almost equivalent to that of IL-3 (Fig. 3). A relatively sustained stimulation, and appearance of an activated Erk-2, were observed upon activation of both D23 and D24 cells by their most potent ligand, namely, NDF, implying that these features may characterize the stronger mito-

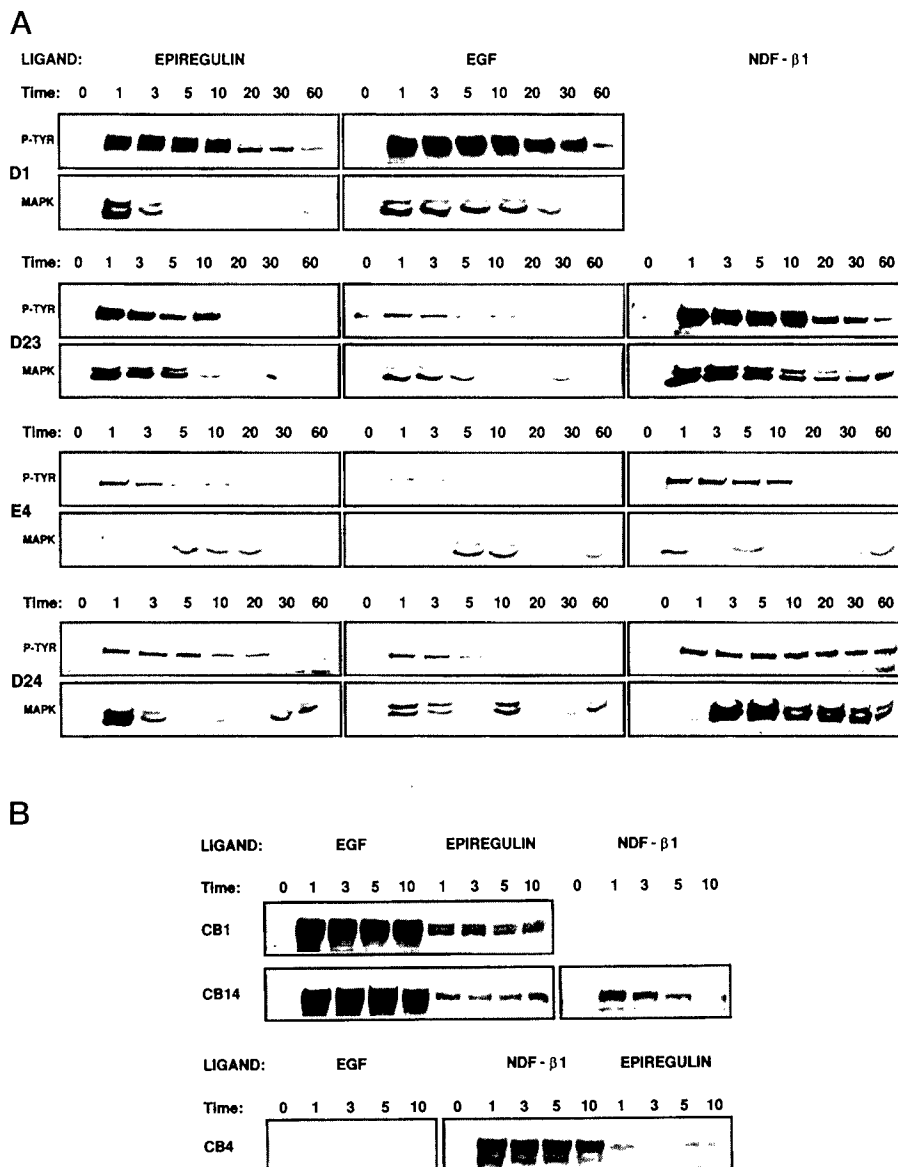


FIG. 4. Kinetics of receptor phosphorylation and MAP kinase activation by epiregulin and other ligands. The following derivatives of 32D cells (D or E series of cell lines, *panel A*), or CHO cells (CB series of cell lines, *panel B*), were incubated at 37 °C for various time intervals (indicated in minutes) with epiregulin (100 ng/ml), EGF (100 ng/ml, except for D23 cells that were treated with 500 ng/ml), or NDF- β 1 (100 ng/ml): D1, CB1, E4, and CB4 cells that singly express ErbB-1 or ErbB-4, respectively, whereas D23, D24, and CB14 cells co-express a combination of the corresponding two receptors. In the end of the incubation period, whole cell lysates were prepared, cleared from debris and nuclei, resolved by gel electrophoresis, and subjected to immunoblotting with either an antibody to phosphotyrosine (P-TYR), or with an antibody specific to the active doubly phosphorylated form of MAPK, as indicated. Derivatives of CHO cells were analyzed only with antibodies to phosphotyrosine. Signal detection was performed by using a chemiluminescence kit.

genic signals. In conclusion, the relative strength of mitogenic signals of EGF-like ligands better correlates with the duration of MAPK activation (especially the modification of Erk-2) than with the intensity of ErbB phosphorylation.

Low Affinity Interaction of Epiregulin with ErbB-1 and Other ErbB Proteins—The relatively weak stimulation of ErbB-1 phosphorylation by epiregulin (D1 *panel* in Fig. 4) suggested low affinity interaction of epiregulin with ErbB-1 on D1 cells. This possibility was addressed by employing two assays: covalent cross-linking of a radiolabeled epiregulin to the surface of ErbB-expressing 32D cell derivatives (Fig. 5), and ligand displacement analyses that were performed with both 32D- and CHO-derived cell lines (Fig. 6). Epiregulin was radiolabeled with 125 I and covalently cross-linked to the surface of 32D cells by using the BS³ covalent cross-linking reagent. The specificity of labeling by epiregulin was evident by the absence of covalent cross-linking to ErbB-2 and ErbB-3, when these receptors were singly expressed (D2 and D3 cells, respectively, Fig. 5), and by displacement of radioactive epiregulin by a large excess of the unlabeled ligand (data not shown). Interestingly, only a very weak signal was observed when radiolabeled epiregulin was covalently cross-linked to cells singly expressing ErbB-1, although these cells displayed a strong cross-linking signal with 125 I-EGF, whose specific radioactivity was comparable to that

of 125 I-epiregulin (Fig. 5). A slightly stronger signal was observed when cells coexpressing ErbB-1 and ErbB-2 were analyzed, implying that the corresponding heterodimer cooperatively interacts with epiregulin. The combination of ErbB-1 with ErbB-3 was less efficient than that of ErbB-1 with ErbB-2, although the numbers of ErbB-1 molecules on D1, D12, and D13 cells were similar. By contrast with ErbB-1, affinity labeling of ErbB-4 in the overexpressing E4 cell line was very efficient in the case of both epiregulin and NDF, but relatively weak labeling was observed with EGF (Fig. 5), in accordance with receptor phosphorylation signals (Fig. 4A). Similar observations were made with the D4 and CB4 cell lines (data not shown). Interestingly, we were unable to detect covalent cross-linking of epiregulin to cells coexpressing ErbB-3 with either ErbB-2 or ErbB-4 (D23 and D34 lanes in Fig. 5, note that ErbB-4 expression in D24 and D34 cells is approximately 10-fold lower than in E4 cells), although these combinations reacted with NDF. By contrast, the ErbB-2/ErbB-4 combination displayed a clearly detectable signal with 125 I-epiregulin, reflecting the relatively high mitogenic response of D24 cells to epiregulin (Figs. 2A and 3).

We then compared the capacity of epiregulin, as opposed to EGF, to displace a cell-bound radioactive EGF from the surface of 32D or CHO cells singly expressing ErbB-1 (D1 and CB1

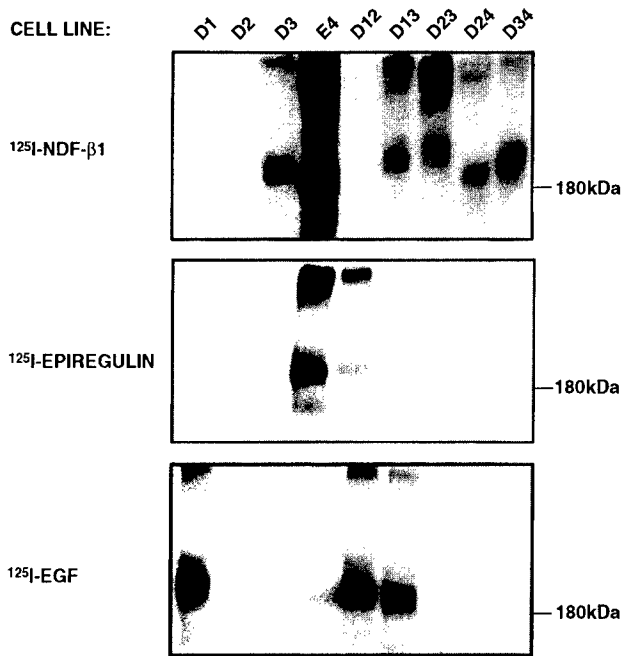


FIG. 5. Covalent cross-linking of radiolabeled epiregulin and other ligands to ErbB-expressing cells. The indicated derivatives of 32D myeloid cells (10^6 cells per lane) were incubated at 4°C with ^{125}I -EGF, ^{125}I -epiregulin, or ^{125}I -NDF- $\beta 1$, each at 100 ng/ml. Following 90 min of incubation, the covalent cross-linking reagent BS³ was added (1 mM final concentration), and cell lysates prepared after an additional 1.5 h of incubation. Lysates were resolved by gel electrophoresis and autoradiography. The location of a 180-kDa molecular weight marker is indicated.

cells, respectively). In contrast with the mitogenic superiority of epiregulin for ErbB-1-expressing 32D cells, the apparent binding affinity of epiregulin, as reflected by the competition curves, was 2 orders of magnitude lower than that of EGF (*D1* and *CB1* panels in Fig. 6, *A* and *B*). The presence of ErbB-4 together with ErbB-1 did not significantly alter the ability of epiregulin to displace EGF from the surface of CB14 cells (Fig. 6*B*), although epiregulin was able to displace, albeit with low efficiency, a surface-bound ^{125}I -NDF from ErbB-4-expressing cells (*D4* or *CB4* cells, Fig. 6, *A* and *B*). The results of ligand displacement experiments that were performed with E4 cells were qualitatively similar (data not shown). NDF displacement by epiregulin, or EGF, was relatively efficient in D24 cells, but only weak competition was detectable in D34 cells, consistent with the relative mitogenic potency of epiregulin for D24 and D34 cells (Figs. 2*A* and 3). Thus, affinity labeling (Fig. 5) and ligand competition analyses (Fig. 6) imply that epiregulin binds cooperatively to the combination of ErbB-2 with ErbB-4. By contrast, only very weak competition between epiregulin and NDF was observed in cells expressing ErbB-3, either alone or in combination with ErbB-2 or ErbB-4 (Fig. 6*A*), implying that ErbB-3, unlike ErbB-4, does not cooperate with ErbB-2 in epiregulin binding. This conclusion is consistent with the absence of a detectable cross-linking signal in D3, D13, and D23 cells (Fig. 5). In light of this inference the results obtained with D23 cells are interesting because epiregulin displayed only a slightly better ability than EGF to displace NDF from these cells, but its mitogenic activity was much stronger than that of EGF (Figs. 2*A* and 3). In conclusion, receptor binding analyses indicated direct interaction between epiregulin and two receptors, ErbB-1 and ErbB-4. Although neither ErbB-3 nor ErbB-2 directly interact with epiregulin, the latter protein significantly cooperates with both direct receptors of epiregulin.

Epiregulin-induced Down-regulation of ErbB-1 Is Defective—The superior mitogenic activity of epiregulin is analogous to

that of TGF α . This latter ligand of ErbB-1 is a better agonist than EGF when tested *in vitro* in mitogenic, angiogenic, and motogenic assays (43, 44). Apparently, the relatively potent activity of TGF α , whose binding affinity is almost identical to that of EGF, is due to the absence of receptor down-regulation, which allows sustained cellular activation (45). To examine the possibility that epiregulin's superiority is due to a defective receptor inactivation process, we exposed CB1 cells to epiregulin, EGF, or TGF α , and determined the extent of disappearance of ErbB-1 from the cell surface. Evidently, whereas EGF induced gradual disappearance of the surface-exposed ErbB-1, neither epiregulin nor TGF α led to a significant change in the level of surface ErbB-1 (Fig. 7), although at the concentrations we used both ligands were more mitogenic than EGF (Fig. 2*A*, and data not shown). In experiments that are not presented we found that the difference in receptor down-regulation was not due to defective endocytosis of epiregulin, whose rate of internalization was comparable to that of EGF and TGF α . This observation raised the possibility that unlike EGF, which directs ErbB-1 to degradation in lysosomes, epiregulin binding to ErbB-1 is followed by receptor recycling, a route taken by the TGF α -driven ErbB-1 (45, 46). This notion was supported by an experiment that tested the effect of monensin, a well characterized inhibitor of receptor recycling (47), on down-regulation of ErbB-1. In the presence of the carboxylic ionophore both epiregulin and TGF α induced significant down-regulation of ErbB-1, but this compound was ineffective on the extensive down-regulation that was induced by EGF (Fig. 7, and data not shown). In conclusion, the relatively strong biological action of epiregulin through ErbB-1 may be due to continuous recycling of ErbB-1 back to the cell surface, thus allowing prolongation of epiregulin signaling.

DISCUSSION

The evolutionary pathway of the ErbB signaling module, from worms (48) and flies (49) to mammals, indicates that duplication of genes encoding EGF-like ligands preceded multiplication of receptor-encoding genes. Despite multiplicity of ligands and receptors, it is clear that the downstream signaling mechanisms, namely a linear cascade leading to MAPK activation, has been conserved. Thus, to gain functional diversity, variations on the common theme of ligand-ErbB-MAPK evolved throughout evolution. Examination of the interactions between one of the mammalian ErbB ligands, epiregulin, and various combinations of the four ErbB proteins uncovered two novel features of the evolved module, that are schematically presented in Fig. 8. First, epiregulin is a broad-specificity ligand that activates all eight ligand-stimulatable combinations of ErbBs. Second, despite its extremely low affinity, signaling by epiregulin is more potent than the bioactivity of a high affinity ligand, namely, EGF. The mechanisms underlying these two features, and their functional implications, are discussed below.

Pan-ErbB Specificity of Epiregulin—The four mammalian ErbB proteins can form 10 homo- and heterodimeric complexes, including an ErbB-3 homodimer, which is biologically inactive (29), and an ErbB-2 homodimer whose formation may be driven by receptor overexpression (50), or by a transmembrane oncogenic mutation (51). Epiregulin can signal through all but these two homodimeric combinations of ErbBs (Fig. 8). This broad specificity is unique; no other EGF-like ligand has such a wide selection of receptors. However, due to its broad selectivity, none of the receptors of epiregulin binds it with high affinity (Figs. 5 and 6).

One of the most surprising observations made in the course of the present study is the ability of both epiregulin and EGF to activate ErbB-4 when this receptor is singly expressed. This

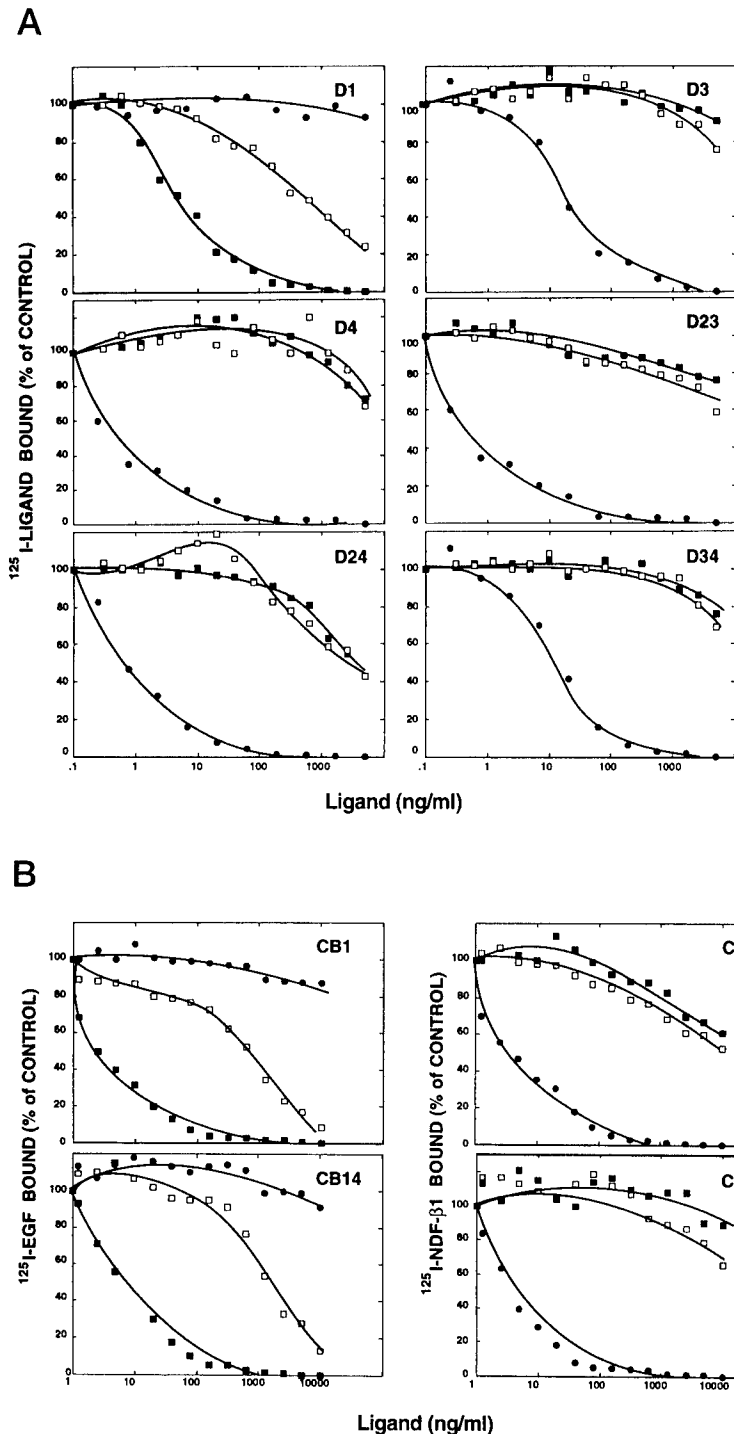


FIG. 6. Binding of epiregulin to cell lines expressing specific ErbB proteins and their combinations. Ligand displacement analyses were performed with derivatives of 32D myeloid cells (D series of cell lines, *panel A*), or with CHO cells expressing ErbB-1, ErbB-4, or their combinations (CB series of cell lines, *panel B*). Either radiolabeled EGF (*D1*, *CB1*, and the left-hand *CB14* panel) or radioactive NDF- β 1 (*D3*, *D4*, *D23*, *D24*, *D34*, *CB4*, and the right-hand *CB14* panel) were used. Cells (10^6) were incubated for 2 h at 4 °C with the radiolabeled ligand (1 ng/ml) in the presence of increasing concentrations of an unlabeled epiregulin (open squares), EGF (closed squares), or NDF- β 1 (closed circles). Each data point represents the mean (less than 10% variation) of two determinations.

observation is reminiscent of several recent reports that identified betacellulin (9) and heparin-binding EGF (10, 11) as ligands of ErbB-4. Conceivably, ErbB-1 and ErbB-4 share some structural features at their ligand-binding sites, thus defining a subgroup of direct ErbB-1 ligands, including EGF, betacellulin, and heparin-binding EGF, but excluding TGF α and amphiregulin, as ligands with dual receptor specificity. Nevertheless, like all other interactions of epiregulin, binding to ErbB-4 is characterized by very low affinity; the corresponding dissociation constant is estimated to be in the micromolar range (*D4* and *CB4* panels in Fig. 6). The affinity of the other direct receptor of epiregulin, ErbB-1, is only 10-fold better, much higher than the nanomolar or lower apparent K_d of EGF or NDF binding to their direct receptors (Fig. 6A). However, re-

ceptor combinations containing ErbB-1 and ErbB-4 are not the only receptors for epiregulin; although this ligand does not interact with isolated components of the ErbB-2/ErbB-3 heterodimer, it can efficiently stimulate the respective receptor combination (*D23* panels in Figs. 2A and 3). This is probably mediated by an extremely low affinity of epiregulin to ErbB-3 (*D3* panel in Fig. 6), and by a cooperative effect of the coexpressed ErbB-2. This effect of the ligand-less ErbB-2 is extended to heterodimers containing the direct epiregulin receptors, namely ErbB-1 and ErbB-4; cooperativity is exemplified by the relatively strong binding of epiregulin to cells coexpressing ErbB-1 and ErbB-2 (but not to cells co-expressing ErbB-1 and ErbB-3, Fig. 5), and by the ability of ErbB-2 to augment epiregulin binding to ErbB-4 (compare *D4* and *D24* panels in

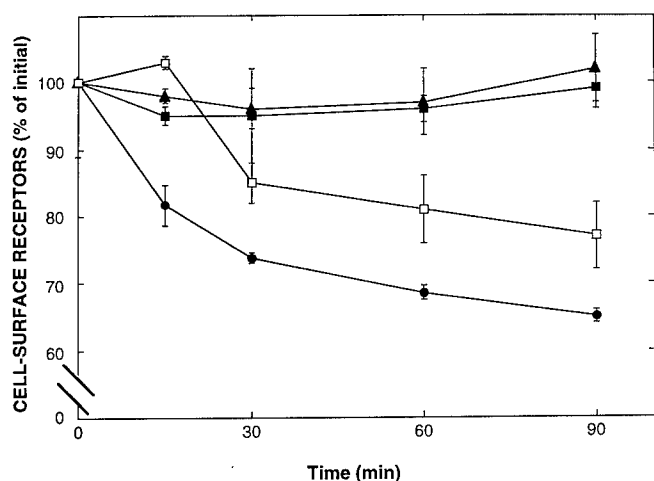


FIG. 7. **Epiregulin-induced down-regulation of ErbB-1.** CB1 cells were grown to 80% confluence in 24-well plates, rinsed with binding buffer, and incubated at 37 °C for the indicated time intervals with one of the following ligands (each at 1 ng/ml): epiregulin (closed squares), EGF (circles), or TGF- α (triangles). Sister epiregulin-treated cells were similarly incubated, except that monensin (0.1 mM) was added to the medium (open squares). Thereafter, monolayers were rinsed twice with binding buffer, followed by a 7-min long incubation with a low pH stripping buffer that removes surface-bound ligands. The level of surface receptors, relative to the number of ligand-binding sites present before down-regulation, was determined by incubating cells for 1.5 h at 4 °C with radiolabeled EGF. The results are expressed as the average fraction and range (bars) of the original binding sites that remained on the cell surface after exposure to the non-labeled ligand at 37 °C.

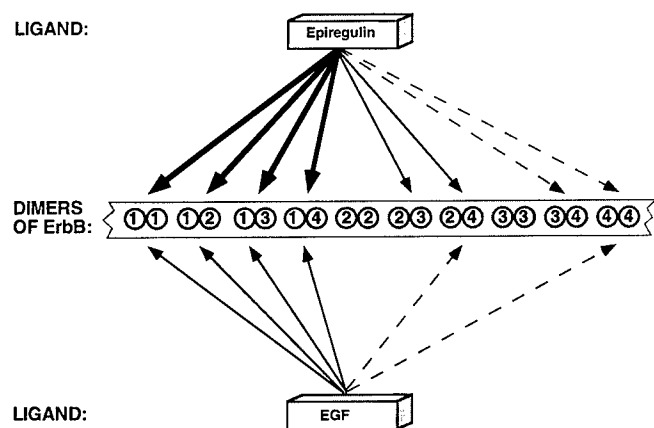


FIG. 8. **Summary of epiregulin-receptor interactions.** The horizontal gray bar represents the plasma membrane, and the 10 possible receptor dimers are shown schematically as double circular structures. Specific ErbB proteins are identified by their numbers. Two ErbB ligands, epiregulin and EGF, are compared and their relative strength of signaling, as revealed by using an IL-3-dependent series of cell lines, are represented by the thickness of the corresponding arrows. Broken arrows indicate very low bioactivity. For simplicity, the ability of EGF to stimulate an ErbB-2/ErbB-3 heterodimer at very high ligand concentrations is not represented. Note that no ligand binds with high affinity to ErbB-2 homodimers. Because no 32D cell derivative co-expressing ErbB-1 and ErbB-4 has been established, the data related to this heterodimeric combination was inferred from experiments with transfected CHO cells. All other receptor combinations were examined in 32D cell derivatives.

Fig. 6). This binding effect is translated to enhanced signaling by the ErbB-2/ErbB-4 heterodimer relative to the ErbB-4 homodimer, and is apparently more relevant to epiregulin than to EGF (compare *E4* and *D24* panels in Fig. 2A). The mechanism underlying signal amplification by ErbB-2, a process that is significant to tumors overexpressing this receptor, has been previously attributed to its ability to decelerate dissociation of

NDF and EGF from ErbB-2-containing heterodimers (25, 52). The present study apparently extends this mechanism to epiregulin.

How does epiregulin recognize all six heterodimeric complexes of ErbBs? According to a ligand bivalence model (12), a notion supported by recent affinity labeling studies (53), and by measurements of the stoichiometry of ligand-receptor interactions in solution (54), epiregulin carries a high affinity binding site whose specificity is limited to ErbB-1 and ErbB-4. Another site that is structurally distinct and may localize to the C-terminal half of the ligand, binds with broad specificity but low affinity to other ErbB proteins, including ErbB-1 and ErbB-4 (thus allowing homodimer formation), as well as to ErbB-2 and ErbB-3, to confer heterodimer formation. Nevertheless, as is the case with EGF and NDF, the putative "low-affinity/broad-specificity" site of epiregulin apparently prefers ErbB-2 over other receptors. This model explains how ErbB-2 augments epiregulin signaling through the ErbB-2/ErbB-3 and ErbB-2/ErbB-4 heterodimers.

Mechanism of Signaling Superiority of Low Affinity Ligand-ErbB Interactions—In their original analysis of epiregulin interactions with various cell types, Toyoda and collaborators (21) found that this ligand was more mitogenic than EGF for several types of normal cells, although epiregulin binding to cells of another type (the A-431 epidermoid carcinoma line) displayed a 10-fold lower affinity. Potentially, this discrepancy could be due to the different repertoires of ErbB proteins expressed on the surface of the different lines of cultured cells that these authors examined. However, our studies with engineered myeloid cells excluded this possibility, because epiregulin's superiority was retained also by cells singly expressing ErbB-1. In fact, our results extend the discrepancy between binding affinity and bioactivity to signaling through ErbB-4. Thus, epiregulin is a relatively potent stimulator of mitogenesis through both ErbB-1 and ErbB-4, despite being a very low affinity ligand of these two receptors (*D1* and *E4* panels in Figs. 2A and 6A). The observation that ErbB-1 phosphorylation by epiregulin is weaker than the effect of EGF (Fig. 4A), implies that receptor activation is not the sole determinant of signaling potency. Instead, differences in the inactivation process may be critical: apart from differential recruitment of both tyrosine-specific phosphatases (55) and the negative regulator c-Cbl (56), endocytosis of ligand-receptor complexes is a major process that leads to inactivation of growth factor signaling (reviewed in Ref. 57). Our initial studies of this aspect of epiregulin's action indicated that this ligand, unlike EGF, mediates limited, if any, down-regulation of ErbB-1 (Fig. 7). Additional analyses raised the possibility that epiregulin undergoes internalization, but its receptor rapidly recycles to the cell surface (Fig. 7). Presumably, the very low affinity of epiregulin to ErbB-1 is insufficient to direct this receptor to lysosomal degradation, either because phosphorylation on tyrosine residues, which is essential for rapid internalization (58), is relatively inefficient, or because the ligand dissociates very rapidly. It is relevant that mutations of another receptor, that stabilize ligand-receptor interactions at the moderately acidic conditions of early endosomes, accelerate receptor degradation and prevent recycling (59, 60), indicating that the strength of ligand binding is critical for receptor routing. This mechanism of epiregulin/ErbB-1 interactions is expected to promote a relatively weak level of receptor activation, but due to receptor recycling, repeated association-dissociation cycles may result in prolongation of signaling. In support of this model, we observed an overall lower activation of MAPK by epiregulin, but this was more prolonged than in the case of EGF (*D1* panel in Fig. 4A). Variations of the proposed mechanism have previ-

ously been reported: in the case of TGF α , whose binding affinity is comparable to that of EGF, the more rapid dissociation of the ligand-receptor complex in an acidic endosomal compartment drives ErbB-1 to recycling (45). This is contrasted with the lysosomal destination taken by an EGF-bound ErbB-1. As a result, signaling by TGF α is often more potent than that of EGF. An even closer example is provided by a mutant of EGF that was engineered to enhance the mitogenic potency of the growth factor for biotechnological applications (61). This mutant achieved mitogenic superiority through a combination of a 50-fold lower affinity, longer retention in culture supernatants, and a very limited receptor down-regulation.

In addition to the question how wide is the relevance of our findings to other growth factors whose binding affinities are very low, several other interesting questions are left open. The exceptionally broad specificity of epiregulin joins other observations that collectively imply non-redundancy of the multiple EGF-like ligands (reviewed in Ref. 62). Evidently, each ligand differs from other members of its family by a unique preference for certain ErbB proteins. This, however, does not explain how different ligands mediate mitogenesis on some cells, but differentiation (37), survival (63), or cell motility (10) on other types of cells, although in all cases the MAPK pathway is recruited. Even more difficult to reason is the inhibitory activity of epiregulin on certain epithelial cell lines (21), because all of its receptors turned out to be stimulatory for myeloid cells (Figs. 2A and 3). Perhaps a cell type-specific component lying downstream of ErbBs determines the nature of cellular response. Another puzzling issue is the contrast between the broad selectivity of epiregulin for ErbBs, and its very limited pattern of expression (23). This and other questions will require *in vivo* studies of epiregulin's physiological role.

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Pathogenic poxviruses reveal viral strategies to exploit the ErbB signaling network

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Virulence of poxviruses, the causative agents of smallpox, depends on virus-encoded growth factors related to the mammalian epidermal growth factor (EGF). Here we report that the growth factors of Shope fibroma virus, Myxoma virus and vaccinia virus (SFGF, MGF and VGF) display unique patterns of specificity to ErbB receptor tyrosine kinases; whereas SFGF is a broad-specificity ligand, VGF binds primarily to ErbB-1 homodimers, and the exclusive receptor for MGF is a heterodimer comprised of ErbB-2 and ErbB-3. In spite of 10- to 1000-fold lower binding affinity to their respective receptors, the viral ligands are mitogenically equivalent or even more potent than their mammalian counterparts. This remarkable enhancement of cell growth is due to attenuation of receptor degradation and ubiquitination, which leads to sustained signal transduction. Our results imply that signal potentiation and precise targeting to specific receptor combinations contribute to cell transformation at sites of poxvirus infection, and they underscore the importance of the often ignored low-affinity ligand-receptor interactions.

Keywords: DNA virus/growth factor/oncogene/signal transduction/tyrosine kinase

Introduction

ErbB-1/HER1 was the first transmembrane tyrosine kinase whose full primary structure was uncovered through molecular cloning (Ullrich *et al.*, 1984). Along with the isolation of additional growth factor receptors harboring an intrinsic tyrosine kinase activity, and their classification into groups of two to 15 structurally related proteins (reviewed in van der Geer *et al.*, 1994), additional members of the ErbB family were discovered. Unlike ErbB-1, which binds at least seven mammalian growth factors, whose prototype is the epidermal growth factor (EGF), no known ligand binds to ErbB-2 with high affinity. The ligands for the two other members of the family, ErbB-3 and ErbB-4, include three families of alternatively spliced growth

factors, collectively called neuregulins (reviewed in Burden and Yarden, 1997). Attempts to understand the role of ErbB-2 led to the realization that this member of the family functions as a shared signaling subunit that decelerates the rate of ligand dissociation from ErbB-2-containing heterodimeric complexes (Karunagaran *et al.*, 1996). Apparently, each of the many EGF-like ligands of ErbBs acts as a bivalent molecule that binds to a primary receptor through one site of the molecule; binding of a second receptor to the other site of the ligand enables homo- or hetero-dimerization of ErbBs (Tzahar *et al.*, 1997).

Binding of an EGF-like ligand to an ErbB protein initiates a signaling cascade that culminates in recruitment of the mitogen-activated protein kinase (MAPK) pathway and results in growth or differentiation signals (reviewed in Alroy and Yarden, 1997). This pathway is conserved through evolution from worms to mammals (Kornfeld, 1997) and mutations along this pathway frequently lead to aberrant growth and malignancy. Examples include an oncogenic viral form of ErbB that induces erythroblastomas and sarcomas in birds (Downward *et al.*, 1984), a chemically induced mutant of ErbB-2 that promotes tumors in the nervous system of rodents (Bargmann *et al.*, 1986), amplification of the *erbB-2* gene in several types of human adenocarcinoma (Slamon *et al.*, 1989), and autocrine production of the transforming growth factor α (TGF α), one of the ligands of ErbB-1, by virally and chemically transformed cells (reviewed in Salomon *et al.*, 1995). Significantly less is known about another autocrine loop, in which the activated growth factor genes are encoded by the invading virus, rather than by the host cell. These DNA viruses, collectively called poxviruses, are the largest of all animal viruses (reviewed in Buller and Palumbo, 1991). Poxviruses infect a wide range of species and produce remarkably different pathologies. Despite this heterogeneity, most if not all viral strains encode EGF-like growth factors that are not essential for viral replication. However, genetic inactivation experiments attributed an essential role to these secreted molecules in enhancement of virulence and stimulation of cell proliferation at the primary site of infection (McFadden *et al.*, 1996).

The EGF-like factors of only three poxviruses have been isolated: vaccinia growth factor (VGF) is synthesized after infection with the cytolytic vaccinia virus as a transmembrane precursor glycoprotein (Blomquist *et al.*, 1984; Brown *et al.*, 1985; Stroobant *et al.*, 1985). The tumorigenic viruses Myxoma virus and Shope fibroma virus encode secreted peptides, MGF and SFGF, respectively, that share 80% amino acid homology, compared with only 34–37% homology to VGF (Chang *et al.*, 1987; Upton *et al.*, 1987). Synthetic analogs of the three growth factors were synthesized and found to interact with ErbB-1,

although the affinity of interaction and the cellular outcome displayed variation and dependence on cell type (Lin *et al.*, 1988, 1990, 1991). Because these studies were performed before the ErbB family was extended to include ErbB-3 and ErbB-4, and only recently has the significance of inter-receptor interactions been fully appreciated, we hypothesized that virus-specific cytopathological landmarks, as well as cell-type specificity of infection, may be due to activation of ErbB proteins other than the EGF receptor. Here we demonstrate that each of the three viral ligands is characterized by a distinct ErbB specificity; whereas SFGF is a broad-specificity ligand that activates all ErbB-1-containing receptor combinations, in addition to the potent ErbB-2/ErbB-3 heterodimer, MGF is strictly selective to the latter receptor complex, and VGF appears to interact preferentially with ErbB-1 homodimers. Remarkably, although the viral ligands bind to the respective receptors with an affinity that is up to 1000-fold weaker than that of the relevant mammalian growth factors, their proliferative signals are similar or even higher. The underlying mechanism appears to involve attenuation of the rapid inactivation processes that are normally coupled to stimulation of signal transduction by receptor tyrosine kinases.

Results

The three viral EGF-like growth factors differ in potency and in specificity to dimeric combinations of ErbB receptors

It has been noted previously that the three known virus-encoded growth factors differ from EGF in their signaling potency and character, although they all bind to the EGF receptor (ErbB-1) (Lin *et al.*, 1988, 1990, 1991). In order to examine the hypothesis that these differences are due to interaction with ErbB proteins, other than the EGF receptor, we prepared synthetic analogs of the three viral growth factors and evaluated their ability to stimulate receptor phosphorylation in two mammary cancer cells: MDA-MB453 cells which express high levels of ErbB-2 and moderate levels of ErbB-3, but do not express ErbB-1, and MDA-MB468 cells which express high levels of ErbB-1 and moderate levels of ErbB-3, but do not express ErbB-2 (Kraus *et al.*, 1987; data not shown). For control we synthesized and similarly tested the following ligands: epiregulin (Toyoda *et al.*, 1995); EGF (47 amino acids long); and human NDF/neuregulin (a 65 amino acid-long β isoform, denoted HRG β 65; Barbacci *et al.*, 1995). Of the tested peptides, MGF and the long analog of NDF/neuregulin were most active in stimulating receptor phosphorylation in the absence of ErbB-1 (MDA-MB453 cells), but EGF and VGF were inactive (Figure 1A). In contrast, all peptides except MGF were active on cells that express no ErbB-2 (MDA-MB468 cells, Figure 1B). These results suggested that the three viral peptides differ in their specificity to ErbB proteins. This possibility was further tested on an engineered series of myeloid cell lines derived from the interleukin-3- (IL-3) dependent 32D cells (Pinkas-Kramarski *et al.*, 1996). This cellular system offers the advantage of testing ligand interaction with individual ErbB proteins, or their combinations, in the absence of endogenous ErbB proteins. Moreover, its dependence on IL-3 for survival renders this series of

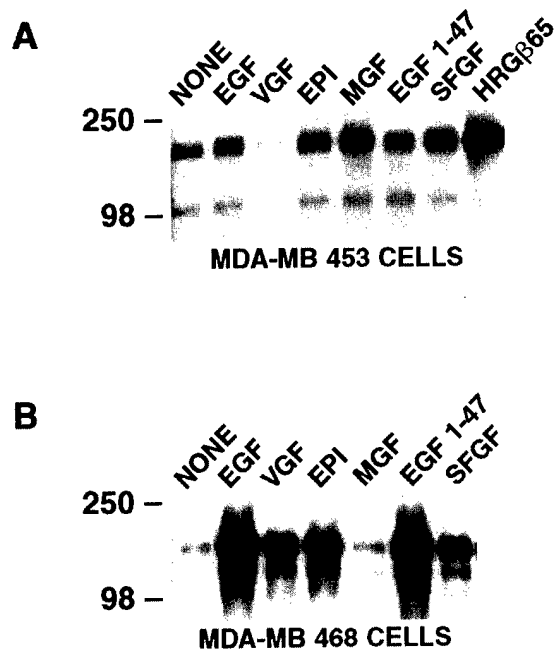


Fig. 1. Stimulation of receptor phosphorylation by EGF-like viral peptides in mammary cells. MDA-MB453 cells (A) and MDA-MB468 cells (B) grown in 48-well plates were stimulated by 50 nM (A) or 5 nM (B) of the indicated peptide for 5 min at 37°C. Total cell lysates were prepared and analyzed by immunoblotting for phosphotyrosine as described (Moyer *et al.*, 1997). All peptides used were synthetic, except for EGF (recombinant, Sigma). EPI indicates epiregulin, and HRG β 65 a 65 amino acid-long fragment of neuregulin (Barbacci *et al.*, 1995). The locations of molecular weight marker proteins are indicated in kDa. The results shown are representative of two independent experiments.

cells very sensitive to growth signals when IL-3 is omitted from the medium. Incubation of the viral growth factors with cells singly expressing ErbB-1 (D1 cells) revealed remarkable differences in the potency of the three growth factors, whereas VGF signaling was comparable to the mitogenic effect of EGF, MGF and SFGF emerged as the least and the most potent mitogens, respectively (Figure 2, D1 panel). Interestingly, not only was SFGF stimulatory at relatively low concentrations, its maximal mitogenic effect was much higher than the mitogenic action of all other growth factors we tested. Examination of cells singly expressing ErbB-2 (D2 cells) or ErbB-3 (D3 cells) revealed that no virus-encoded ligand acts as an agonist for these receptors (Figure 2; data not shown). Whereas lack of effect on ErbB-3 is likely to be due to the inactive kinase domain of this receptor (Guy *et al.*, 1994), the inability of the viral ligands to stimulate ErbB-2 is in line with the notion that this receptor acts exclusively as a shared signaling subunit of other ErbB proteins (Tzahar and Yarden, 1998). Cells singly expressing ErbB-4 (D4 cells) at a very high level responded to low concentrations of both NDF and EGF, in line with a previous report that documented binding of EGF to ErbB-4 (Shelly *et al.*, 1998), but neither viral ligand was active at concentrations <1 ng/ml (Figure 2, D4 panel). Interestingly, the activity of the ErbB-1 superagonist, namely SFGF, on ErbB-4-expressing cells was relatively low, whereas VGF emerged as the least mitogenic factor for these cells, although its effect on D1 cells was equivalent to or better than the action of EGF. Taken together, these results suggest that

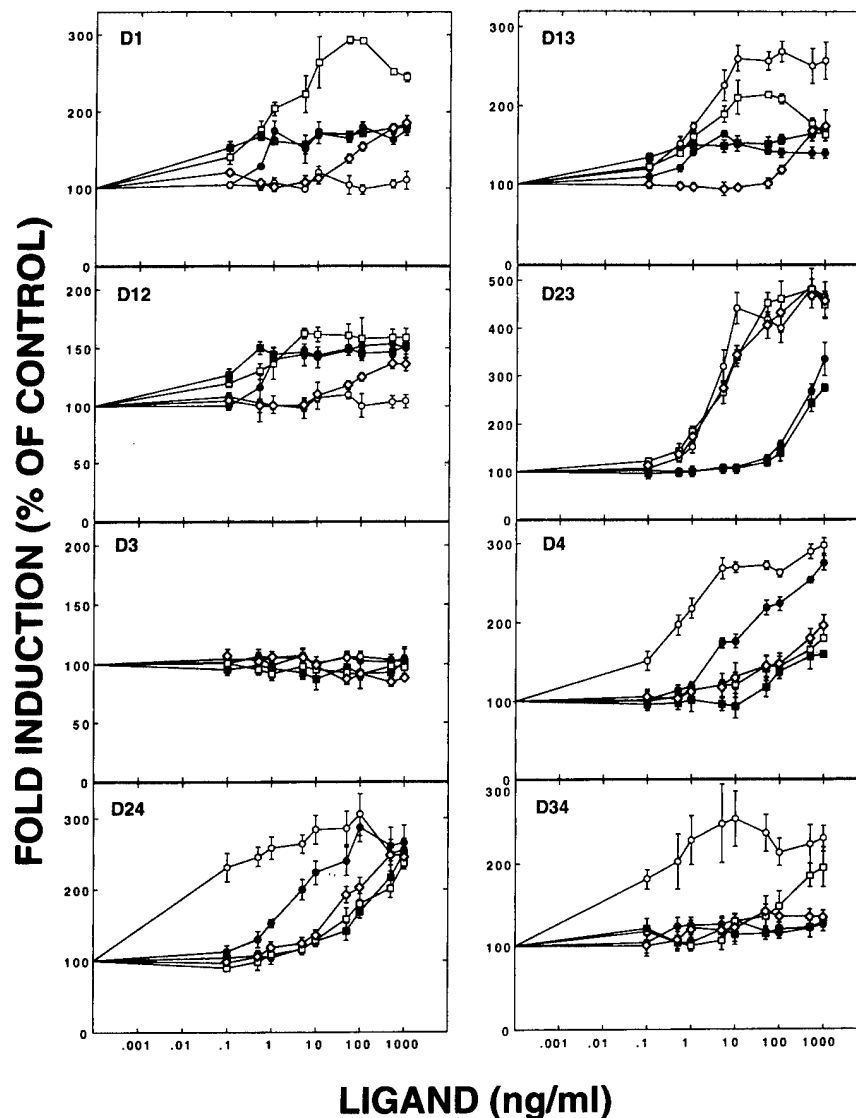


Fig. 2. Proliferative responses of ErbB-expressing derivatives of 32D cells to viral growth factors. The indicated sublines of 32D cells were tested for cell proliferation using the MTT assay. D1 cells singly express ErbB-1, whereas D12 and D13 express ErbB-1 together with ErbB-2, or with ErbB-3 (respectively). The other cell lines were named accordingly after their ErbB repertoires. Cells were plated at a density of 5×10^5 cells/ml in media deprived of IL-3 but containing serial dilutions of the following growth factors: EGF (●); NDF (○); SFGF (□); VGF (■); and MGF (◇). The MTT assay was performed 24 h after growth factor addition. The results are presented as fold induction relative to control untreated cells, and are the mean \pm S.D. (bars) of four determinations. Each experiment was repeated twice with substantially similar results.

the three viral growth factors are distinct from each other, as well as from EGF, in terms of their ability to interact with ErbB-1 and ErbB-4.

Co-expression of two ErbB proteins in 32D cells permits examination of the relative signaling potency of receptor heterodimers, which are thought to be the major signaling complexes *in vivo* (Lee *et al.*, 1995; Riese *et al.*, 1995; Wallasch *et al.*, 1995; Cohen *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996). Expression of ErbB-2 together with ErbB-1 (D12 cells) elevated the basal factor-independent proliferative activity of these cells in line with previous reports (Kokai *et al.*, 1989; Cohen *et al.*, 1996; Zhang *et al.*, 1996), and, therefore, reduced sensitivity of our assay. Nevertheless, similar to the case of D1 cells, the activity of SFGF on D12 cells was higher than that of EGF and MGF, and VGF displayed better or equivalent potency to that of EGF (Figure 2, D12). This relative

order of activity was reflected also in long-term survival experiments, in which D12 cells were deprived of IL-3, but their survival was prolonged, to a varying extent, by the viral growth factors (Figure 3, D12). The superior mitogenic activity of SFGF was reflected also when a combination of ErbB-1 with ErbB-3 was examined (D13 cells): although these cells responded best to NDF, the response to SFGF was greater than to EGF and VGF, and, once again, MGF emerged as the least potent mitogen of the four ligands (compare D1 with D13 in Figures 2 and 3). Surprisingly, however, MGF exerted mitogenic signals almost as potent as those of IL-3, the ultimate growth factor of 32D cells, when cells expressing a combination of the ligand-less receptor, ErbB-2, with the kinase-defective receptor, ErbB-3, were examined; on these cells, denoted D23, MGF acted not only as a potent survival factor, but it significantly stimulated cell proliferation

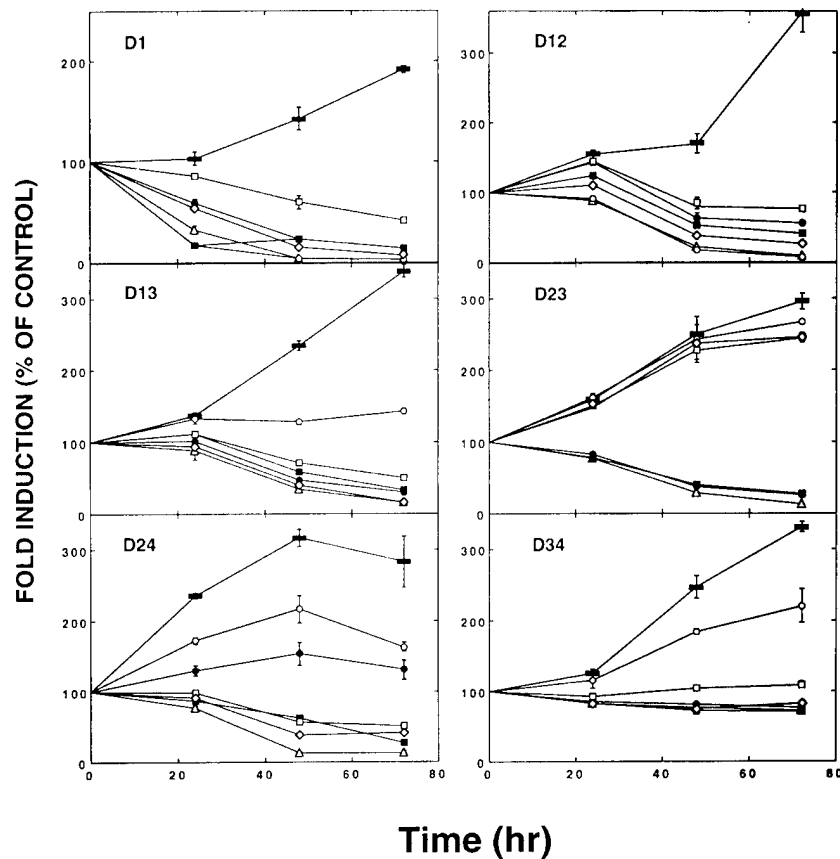


Fig. 3. Ligand-dependent survival of ErbB-expressing cells. The indicated sublines of 32D cells were incubated at a density of 5×10^5 cells/ml with media supplied with IL-3 (■), or with the following growth factors (each at 100 ng/ml): EGF (●); NDF (○); VGF (■); SFGF (□); and MGF (◇). For control, we incubated cells with no factor (open triangles). The extent of cell proliferation was determined daily by using the colorimetric MTT assay. The data presented are the mean \pm S.D. of four determinations. The experiment was repeated twice with similar results.

(D23 in Figures 2 and 3). Likewise, SFGF and NDF displayed potent actions on D23 cells, but EGF and VGF were active only at very high concentrations (>200 ng/ml), in line with two recent reports (Alimandi *et al.*, 1997; Pinkas-Kramarski *et al.*, 1998). It is interesting to note that no other combination of ErbB-2 or ErbB-3 with other receptors was able to potentiate the effect of MGF to the level observed with D23 cells, although this factor was slightly more potent than SFGF on D24 cells, and only very weakly stimulated D34 cells (D24 and D34 in Figures 2 and 3). When compared with either NDF or EGF, MGF emerged as a weak mitogen for all other receptor combinations, including ErbB-1/ErbB-2 and ErbB-1/ErbB-3. In conclusion, our results indicate that the three viral growth factors can stimulate ErbB proteins other than the EGF receptor (ErbB-1), but they differ significantly from one another and from EGF: VGF is as potent as EGF on ErbB-1-containing combinations, but is much weaker than EGF on homo- and hetero-dimers of ErbB-4. On the other hand, SFGF emerges as a superagonist of ErbB-1-containing receptor combinations, but like VGF its ability to stimulate homo- or hetero-dimeric complexes of ErbB-4 is rather weak. The third viral ligand, MGF, exhibits the most narrow selectivity; its action is practically limited to cells co-expressing a combination of ErbB-2 with ErbB-3.

The convergence of signal transduction by all ErbB combinations at the mitogen-activated protein kinase

(MAPK) pathway (Alroy and Yarden, 1997) enabled us to support the results obtained using cell proliferation or survival assays. The various derivatives of 32D cells were incubated for 10 min with a relatively high concentration of each viral ligand, and the state of receptor activation determined by using antibodies to phosphotyrosine. To assay MAPK activation we used a monoclonal antibody that specifically recognizes the active, doubly phosphorylated form of the ERK1 and ERK2 MAPKs (Yung *et al.*, 1997). The results of these analyses are shown in Figure 4. Evidently, ErbB-1 phosphorylation by EGF was greater than the effect of the three viral ligands on cells singly expressing this receptor (D1 cells). In fact, a hyperphosphorylated form of ErbB-1, whose electrophoretic mobility is slower, was most prominent in EGF-treated cells. A similar picture emerged when cells expressing a combination of ErbB-1 with ErbB-3 (D13 cells) were examined. This contrasted with the observation that the mitogenic responses of SFGF and VGF were higher than that of EGF on all ErbB-1-expressing cells we examined (Figures 2 and 3). A better reflection of the relative mitogenic potency was provided by the analysis of MAPK: activation by SFGF, as well as by VGF, was comparable to that observed with EGF, whereas the least mitogenic ligand, MGF, induced only weak signals in D1 and in D13 cells. As expected, however, MGF potently stimulated receptor phosphorylation, as well as MAPK activation, in cells co-expressing ErbB-2 and ErbB-3 (D23 in Figure 4), in

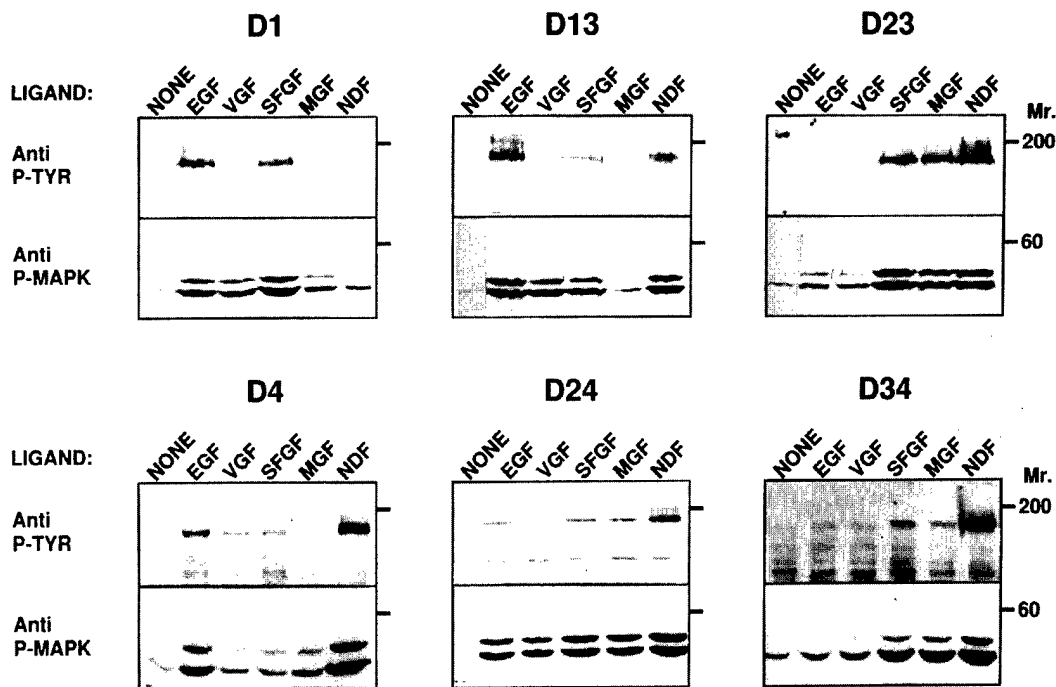


Fig. 4. Receptor phosphorylation and MAPK activation in response to viral ligands. The indicated cell-lines were incubated for 10 min at 22°C with various peptide growth factors (each at 100 ng/ml). Control cultures were incubated with no added factor (NONE). The reaction was terminated by adding hot (95°C) gel sample buffer. The resultant whole cell lysates were subjected to gel electrophoresis, followed by immunoblot analysis with either an anti-phosphotyrosine antibody (PY-20; upper panels), or with antibodies directed to the activated, double phosphorylated form of MAPK (P-MAPK; lower panels). Signals were developed using an ECL kit (Amersham). Note that D4 cells express ~10-fold more ErbB-4 molecules than the D24 and D34 cell lines.

agreement with the potent mitogenic signal observed with D23 cells (D23 in Figures 2 and 3), and the effect of this ligand on mammary cells (Figure 1). Likewise, NDF and SFGF, which acted as potent mitogens on D23 cells, were as active as MGF in the MAPK assay, but the non-mitogenic ligands of D23 cells, namely EGF and VGF, exerted no effect on MAPK. Similarly, analyses of ErbB-4-expressing cells revealed some concordance between MAPK activation and the mitogenic effect of the growth factors we tested. Thus, NDF exerted the most potent effects on both ErbB phosphorylation and MAPK activation in D4, D24 and D34 cells, and the second potent ligand for D4 and D24 cells, namely EGF, induced slightly lower signals (Figure 4). The effects of SFGF and MGF on receptor phosphorylation in ErbB-4-expressing cells were weak compared with NDF, although their ability to stimulate MAPK in these cells was surprisingly high. This raised the possibility that the kinetics, rather than potency, of MAPK activation by the viral ligands is critical for growth signals (see below). In conclusion, receptor phosphorylation and MAPK activation by the viral ligands only partially correlate with the longer lasting growth effects.

Ligand displacement analyses reveal discordance between signaling potency and binding affinities of viral ligands

The large differences in signaling potency that we observed with the three viral ligands, and especially the difference between SFGF and EGF on ErbB-1-expressing cells (D1 in Figure 2), raised the possibility that ligand-binding affinities may explain how the same receptor combination

can generate weak or strong signals in response to ligand binding. Two series of cell lines were used to test this scenario by using ligand-displacement analyses. In addition to the 32D cell derivatives described above, we employed a similar set of Chinese hamster ovary (CHO) cells that express various combinations of the ErbB proteins on a low background of an endogenous hamster ErbB-2 (Tzahar *et al.*, 1996). Due to their adhesion to the substrate, the CHO-derived cells (denoted CB cells), unlike the non-adherent 32D cells, enable more sensitive ligand-binding assays. Competition between increasing concentrations of an unlabeled EGF and a relatively low concentration of ^{125}I -EGF showed that the apparent affinity of this ligand to 32D or CHO cells singly expressing ErbB-1 (D1 and CB1 cells, respectively) was in the range of 0.2–1 nM, but the apparent affinity of VGF was 5- to 7-fold lower (CB1 and D1 panels in Figure 5). Surprisingly, the other two viral ligands, SFGF and MGF, displayed much lower binding affinities: on the basis of the results obtained with CB1 cells we estimate that the affinity of SFGF is 100- to 1000-fold lower than that of EGF, and MGF displayed an even less tight binding to ErbB-1 (CB1 in Figure 5). Similar displacement analyses that used ^{125}I -NDF as a tracer revealed very large differences between the apparent binding affinities of SFGF, MGF and NDF towards cells co-expressing ErbB-2 together with ErbB-3 (CB23 and D23 in Figure 5), although the three ligands were almost equipotent in cell proliferation assays (D23 in Figures 2 and 3). Remarkably, the affinity of MGF was slightly better than that of SFGF, but the affinities of both ligands were 2–3 orders of magnitude lower than the apparent affinity of NDF. Thus, similar to the discrepancy

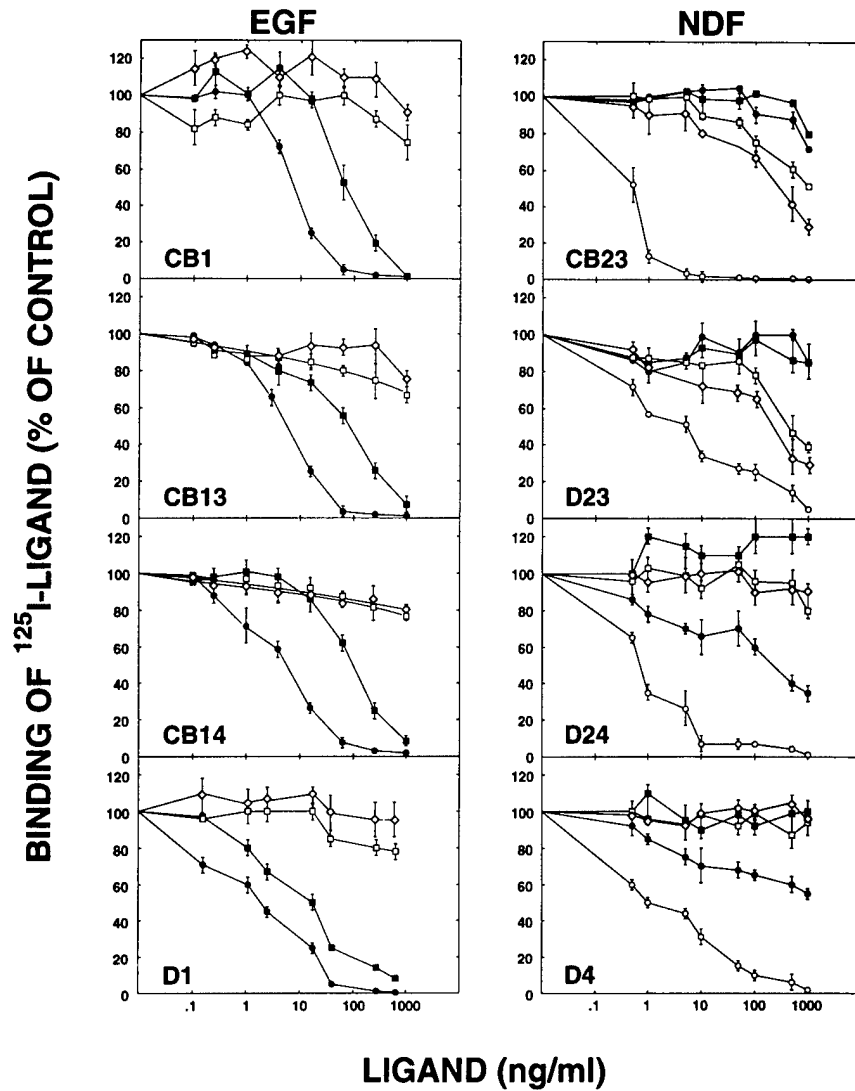


Fig. 5. Displacement of radiolabeled EGF or NDF molecules by viral growth factors. Two engineered series of ErbB-expressing cell lines were used for radioligand displacement analysis. The D cell lines are derivatives of 32D myeloid cells, whereas the CB cell lines were derived from CHO cells by transfecting the respective erbB cDNAs. For example, CB23 cells co-express ErbB-2 and ErbB-3. Cells were incubated for 2 h at 4°C with 1 ng/ml of either ^{125}I -EGF (left column) or ^{125}I -NDF (right column), in the presence of the indicated increasing concentrations of unlabeled EGF (●), NDF (○), VGF (■), SFGF (□) or MGF (◇). Unbound ligands were removed as described under Materials and methods, and cell-associated radioactivity determined by counting gamma irradiation. Each data point represents the mean \pm range (bars) of two determinations. The experiment was repeated twice.

observed in ErbB-1-expressing cells, both SFGF and MGF stimulated D23 cells far better than would be expected on the basis of their relative binding affinities. This discrepancy is in marked contrast with the non-viral ligands we tested, namely EGF and NDF, whose mitogenic potencies well reflected binding affinities. For example, in line with the 10- to 100-fold lower mitogenic potency of EGF relative to NDF, for cells expressing ErbB-4 (either alone or in combination with ErbB-2; Figure 2), this ligand displayed a correspondingly lower binding affinity to erbB-4-expressing cells (D4 and D24 in Figure 5). Once again, despite extremely low affinity of the three viral ligands to ErbB-4 expressing cells (Figure 5), these ligands were nevertheless mitogenic for D4 cells, although at concentrations >10 ng/ml (Figure 2).

Our attempts to determine binding parameters of the three viral ligands by directly using their radiolabeled

forms have failed (data not shown), probably due to the very low affinity of these ligands to all combinations of ErbB proteins. However, blocking ligand dissociation, by using a covalent crosslinking reagent, provided us with a qualitative binding assay, and also permitted examination of the ability of the viral ligands to induce various homo- and hetero-dimers of ErbBs. The results of these affinity-labeling experiments are presented in Figure 6. Evidently, all three ligands labeled monomers and dimers of ErbB-1, as well as both species of ErbB-4, at variable efficiency. Although quantitative comparison of the efficiency of receptor labeling by the three ligands may not reliably reflect their relative binding affinities, due to potential sequence-specific differences in the extent of labeling and chemistry of crosslinking, the ratio of dimers to monomers, and especially the extent of co-immunoprecipitation of the affinity-labeled ErbBs, may be used as

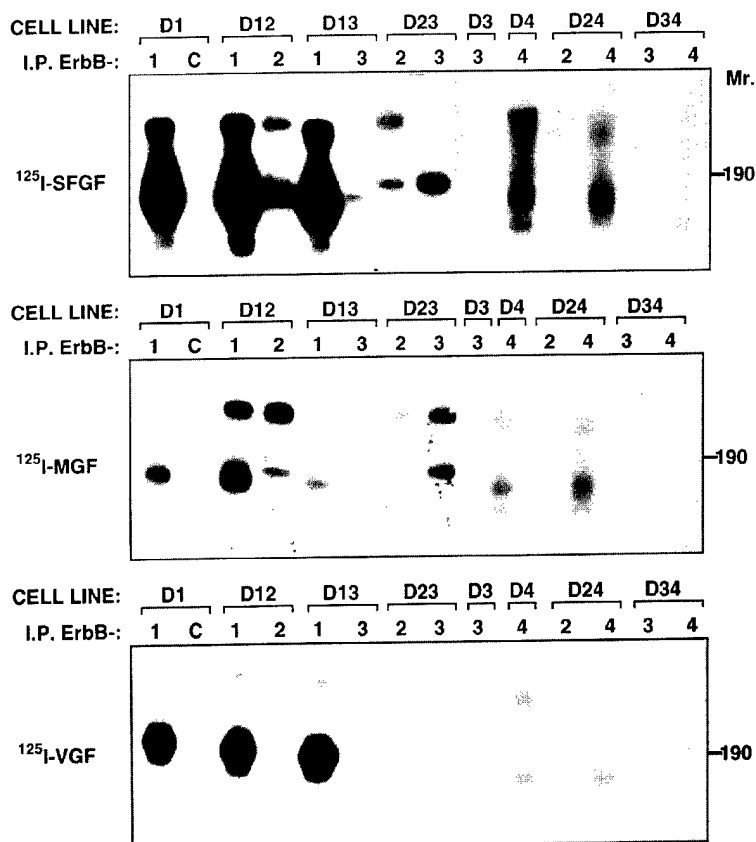


Fig. 6. Covalent crosslinking of radiolabeled viral ligands to 32D cell derivatives expressing individual ErbBs and their combinations. The indicated derivatives of 32D cells (0.5×10^7 per lane) were separately incubated with the three radiolabeled viral peptides (250 ng/ml), as indicated. Following 2 h at 4°C, the covalent crosslinking reagent *bis*(sulfosuccinimidyl)-suberate (BS^3) was added (1 mM) for an additional incubation for 45 min. Then cell lysates were prepared and subjected to immunoprecipitation with antibodies against the indicated ErbB proteins, or with a control antibody, labeled C. The complexes were resolved by gel electrophoresis, followed by autoradiography. Exposure times of the X-ray films were 72, 48 and 36 h, for SFGF, MGF and VGF, respectively. The locations of molecular weight marker proteins are indicated in kDa.

an indicator of ligand-induced receptor interactions. For example, ErbB-2 was best recruited by MGF into heterodimers with ErbB-1 (D12 lanes in Figure 6). It is important to note that no ligand interacted detectably with ErbB-3 when this receptor was present alone (D3 lanes, Figure 6). However, ErbB-3 was quite efficiently labeled by both SFGF and MGF, but not by VGF, when co-expressed with ErbB-2 (D23 lanes in Figure 6). Remarkably, in D23 cells MGF promoted homodimers of ErbB-3, and heterodimers between ErbB-2 and ErbB-3, more efficiently than did SFGF (D23 lanes in Figure 6). Taken together, the superior ability of MGF to engage heterodimers of ErbB-2 with either ErbB-3 or with ErbB-1 implies that this growth factor evolved as a heterodimer-specific ligand. This may explain why MGF displayed very low binding and mitogenicity when tested on cells singly expressing ErbB-1, as opposed to its high activity on the combination ErbB-2/ErbB-3 (D23 in Figures 2 and 3, and CB23 and D23 in Figure 5). By contrast with the heterodimer specificity of MGF, VGF emerged as a ligand that hardly forms heterodimers: practically, only monomeric forms of ErbB-1 were observed in D1, D12 and D13 cells labeled with ^{125}I -VGF. However, although VGF bound very weakly to ErbB-4, it detectably engaged dimers of this receptor (Figure 6), but this led to only low mitogenicity (Figure 2).

SFGF and EGF signal through similar pathways but the viral ligand induces sustained MAPK activation in the nucleus

We next addressed the mechanism that confers signaling superiority to SFGF despite the extremely low affinity of this ligand to ErbB receptors. Two models were examined: according to the first, the viral ligands bind to a site(s) of ErbB-1 that is distinct from the EGF-binding cleft, and therefore these ligands differ in their potency of receptor stimulation. However, covalent crosslinking experiments with D1 cells indicated that a monoclonal antibody (mAb) that selectively binds to the EGF-binding cleft of ErbB-1 (mAb111) inhibited binding of SFGF, as well as that of EGF, to ErbB-1 (Figure 7A). A second ErbB-1-specific antibody, mAb199, affected the binding of neither EGF nor SFGF, implying that the two ligands bind to immunologically indistinguishable sites of ErbB-1. This conclusion was confirmed by using three additional mAbs to the human ErbB-1 (data not shown). According to the second model the viral and the mammalian ligands similarly induce receptor activation, but after binding of SFGF, ErbB-1 couples to a set of signaling proteins that is distinct from the collection of cytoplasmic effectors that are recruited by an EGF-bound ErbB-1. Several downstream effectors of ErbB-1 were examined using immunoblot analysis of whole lysates derived from ligand-stimulated D1 cells. Two examples

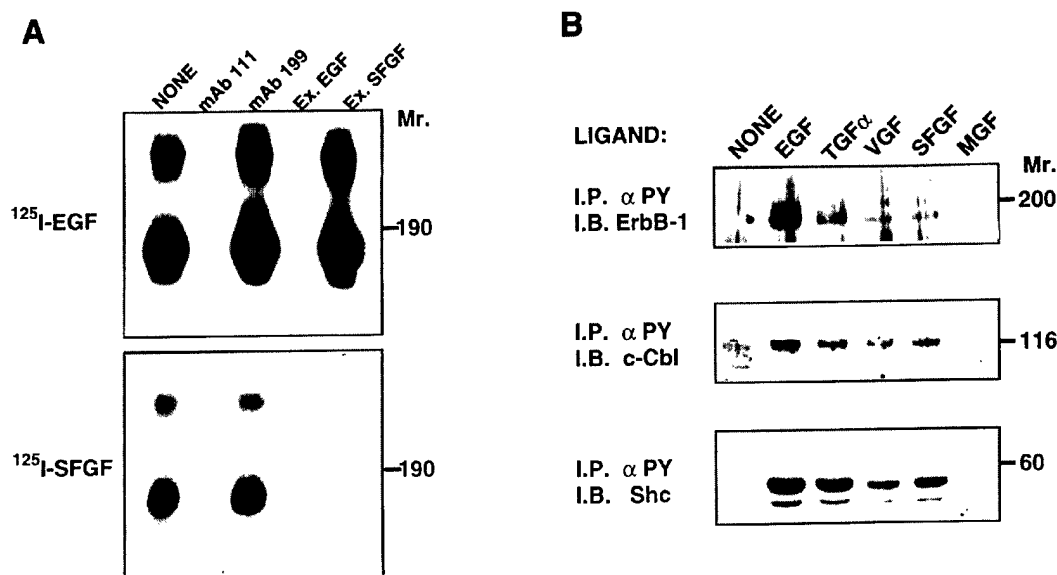


Fig. 7. Comparison of signaling by viral and mammalian ligands. (A) Effect of mAbs on binding of SFGF and EGF to ErbB-1. Covalent crosslinking of radiolabeled EGF (upper panel) or a radiolabeled SFGF (lower panel) to D1 cells was performed as described in the legend to Figure 6, except that incubation with the radiolabeled ligands was performed in the presence of one of the following mAbs (each at 20 $\mu\text{g/ml}$): mAb111 that inhibits EGF binding to ErbB-1, and mAb199 that does not affect the binding of EGF to ErbB-1. For control, cells were incubated in the absence of either mAb (lanes labeled NONE), or in the presence of a 100-fold higher concentration of the indicated unlabeled ligands (Ex. EGF or Ex. SFGF). Following covalent crosslinking with BS^3 and preparation of whole cell lysates, ErbB-1 was subjected to immunoprecipitation using a rabbit antiserum directed against a synthetic peptide derived from the C-terminus of ErbB-1. Note that the specific activities of the two radiolabeled ligands were comparable, but nevertheless labeling with EGF was more intense. (B) Recruitment of cytoplasmic signaling proteins by ErbB-1 in response to ligand stimulation. D1 cells (0.1×10^6 cells/lane) singly expressing ErbB-1 on the cellular background of the 32D cell line were incubated for 10 min at 37°C with the indicated ligands, each at 60 ng/ml. Control cultures were incubated with no added factor (NONE). Whole cell lysates were then prepared and subjected to immunoprecipitation (I.P.) with an anti-phosphotyrosine mAb. The resulting protein blots were subjected to an immunoblot analysis (I.B.) with antibodies to ErbB-1, c-Cbl, or Shc, as indicated.

are shown in Figure 7B: the c-Cbl adaptor protein, whose engagement is stimulated by ErbB-1, but not by other ErbB proteins (Levkowitz *et al.*, 1996), and Shc, a common effector of all ErbB proteins (Culouscou *et al.*, 1995). Evidently, both protein transducers underwent tyrosine phosphorylation in response to the viral ligands SFGF and VGF, but MGF hardly affected them, in accordance with the very weak mitogenic effect of this ligand on cells singly expressing ErbB-1 (Figure 2). Two well-characterized mammalian ErbB-1 ligands, EGF and TGF α , led to comparable phosphorylation of c-Cbl and Shc, although receptor activation by these ligands was significantly higher than the action of the three viral ligands (Figure 7B, upper panel). Activation of MAPK, c-Jun N-terminal kinase and p38RK (HOG; reviewed in Davis, 1994), along with several other cytoplasmic proteins, was another feature shared by SFGF, VGF, EGF and TGF α (data not shown), suggesting that overlapping, if not identical, sets of signaling proteins are recruited by all ErbB-1 ligands.

Because the signaling pathways activated by SFGF and EGF appear similar, and on the other hand signaling by all ErbB proteins is funneled into the MAPK pathway (reviewed in Alroy and Yarden, 1997), we considered the possibility that SFGF signaling is unexpectedly potent due to differences in the kinetics of MAPK activation. To test this prediction we stimulated D1 cells with either SFGF or EGF and examined the state of MAPK activation at increasing time intervals. Remarkable differences in the kinetics of MAPK activation were uncovered by this experiment (Figure 8A): whereas EGF induced transient activation of MAPK that peaked at 2–5 min, the enzyme remained in

its active state 1 h after stimulation with SFGF. By contrast, tyrosine phosphorylation of ErbB-1 was much stronger after binding of EGF, but in both cases receptor stimulation reached a peak at ~ 10 min and then declined slowly. We concluded that the relatively weak binding of SFGF to ErbB-1 is followed by a limited stimulation of tyrosine phosphorylation of this receptor, but this is sufficient for sustained and quite potent activation of the MAPK pathway.

It has been shown previously that MAPK activation is followed by rapid translocation of the phosphorylated kinase to the nucleus (Chen *et al.*, 1992; Lenormand *et al.*, 1993), where it stimulates transcription of specific genes. To test the prediction that SFGF more efficiently translocates MAPK to the nucleus than does the mammalian growth factor, we followed the subcellular localization of MAPK by using antibodies specific to the active form of the kinase. Whereas in unstimulated D1 cells only background cellular staining was observed, binding of either EGF or SFGF was followed by rapid appearance of the active form of MAPK in the nucleus (Figure 8B; staining with a DNA-intercalating dye, DAPI, allowed visualization of nuclei). Consistent with the results shown in Figure 8A, immunostaining was more prominent in cells stimulated with SFGF and it lasted for longer.

Receptor ubiquitination and inactivation in response to viral growth factors is attenuated

The observed sustained MAPK activation by SFGF, in combination with the relatively weak receptor phosphorylation that was induced by this ligand, hinted that the process of signal termination, rather than signal activation,

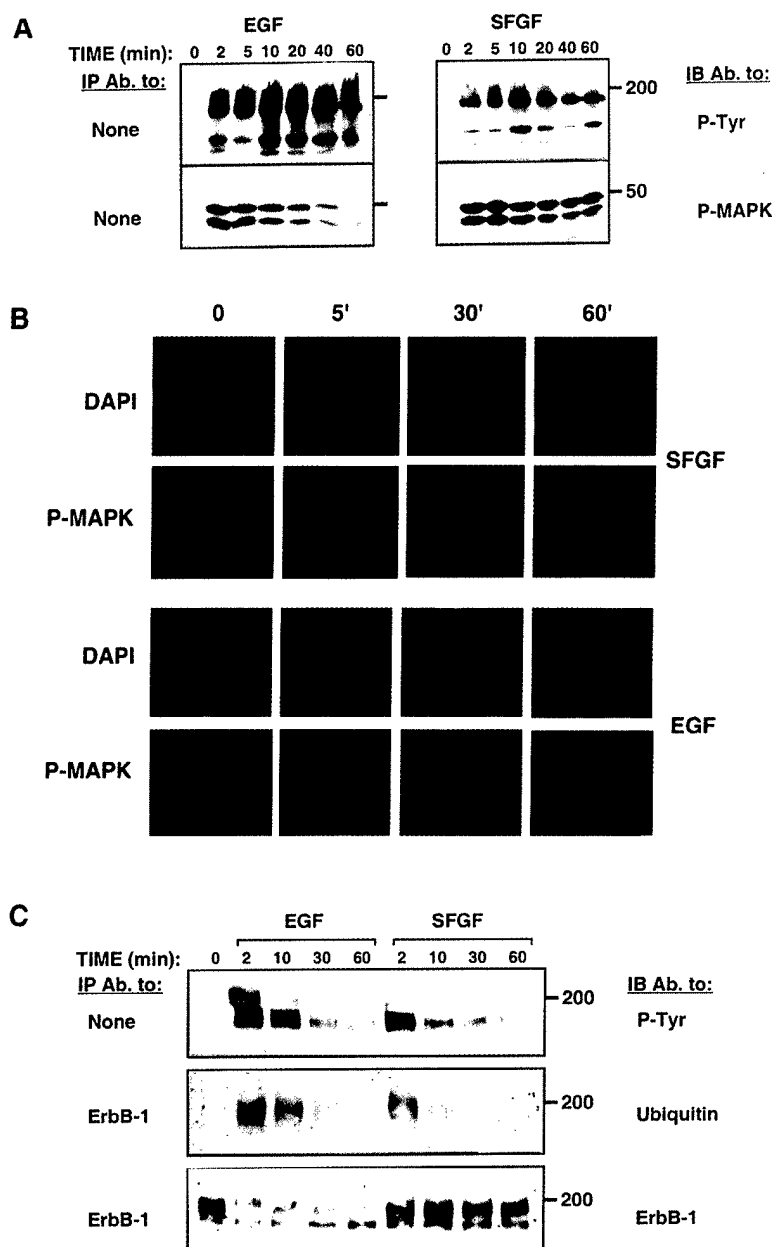


Fig. 8. Time course of ligand-mediated receptor phosphorylation, MAPK activation and retention in the nucleus, and receptor ubiquitination/degradation. **(A)** D1 cells (0.1×10^6 cells/lane) were incubated at 37°C with either SFGF or EGF (each at 50 ng/ml). Following the indicated time intervals, cell stimulation was terminated by adding boiling sample buffer directly on cells. Whole cell lysates were then subjected to immunoblot analysis with either an anti-phosphotyrosine mAb (upper panels), or a mAb directed to the active, doubly phosphorylated form of MAPK (lower panels). The locations of molecular weight marker proteins are indicated in kDa. **(B)** D1 cells (5×10^4) were stimulated at 37°C with EGF or SFGF. Cytospin preparations of these cultures were then fixed and cells permeabilized by incubation in PBS containing Triton X-100 (0.1%). To visualize nuclear DNA, cells were stained with DAPI. Active MAPK was detected by using a mAb specific to the active doubly phosphorylated form. **(C)** D1 cells (10^7 cells/lane) were stimulated at 37°C with EGF or SFGF (50 ng/ml each). Whole cell extracts were prepared at the end of the indicated periods of time and divided into two aliquots. One fraction (20%) was directly subjected to gel electrophoresis, while the other aliquot was subjected to immunoprecipitation (IP) with antibodies to ErbB-1. Gel electrophoresis (7.5% polyacrylamide) of cell lysates or immunoprecipitates was followed by immunoblotting (IB) with antibodies to either phosphotyrosine (P-Tyr) or ubiquitin. The anti-ubiquitin blot was used for re-blotting with a mAb directed to the C-terminal peptide of ErbB-1. Note that alignment of the two lower panels identified the ubiquitinated protein band as the minor upper band of the tyrosine-phosphorylated ErbB-1.

is important for understanding how SFGF potentiates mitogenic signals. Perhaps the most prominent mechanism of receptor inactivation, termed downregulation, is the rapid removal of activated ligand-receptor complexes from the cell surface, and their proteolysis in intracellular vesicular compartments (reviewed in Sorkin and Waters, 1993). We first compared the abilities of SFGF and EGF

to induce degradation of ErbB-1 in D1 cells. Immunoblot analysis of ErbB-1 isolated from ligand-stimulated cells revealed rapid disappearance of the 170 kDa receptor band upon short exposure to EGF (Figure 8C, lower panel). In contrast, SFGF induced only limited receptor degradation, even after long exposure to the viral ligand (Figure 8C, lower panel). Because ErbB-1 degradation

involves elevated ubiquitination, and both ubiquitination and degradation depend on endocytosis of the ligand-receptor complexes (Galcheva-Gargova *et al.*, 1995), we compared the state of receptor ubiquitination by using two methods: electrophoretic resolution of the ubiquitinated species, whose electrophoretic mobility is retarded (Figure 8C, upper panel), and direct identification of receptor-ubiquitin complexes by using anti-ubiquitin antibodies (Figure 8C, middle panel). These analyses indicated that EGF causes rapid appearance of a high molecular weight tyrosine phosphorylated band, which was recognized by anti-ubiquitin antibodies. The appearance of this minor phosphorylated band was significantly lower after cell stimulation by SFGF, and the induction of receptor ubiquitination by this viral ligand was limited and transient compared with that induced by the mammalian ligand. Taken together, the results presented in Figure 8C indicated that receptor ubiquitination and degradation were less efficiently induced by SFGF.

To follow downregulation of ErbB-1 we chose CB1 cells because these adherent cells, unlike 32D derivatives, offer a more sensitive experimental system, yet ligand endocytosis and degradation, as well as sustained MAPK activation, are comparable to the processes exhibited by D1 cells (Pinkas-Kramarski *et al.*, 1996; Tzahar *et al.*, 1996; data not shown). CB1 cells were first incubated at 37°C with the three viral ligands, or with a relatively high concentration of EGF, and the level of ErbB-1 that remained at the cell surface was then determined by performing a binding assay with a radiolabeled derivative of EGF. The results of this experiment revealed that whereas most (>70%) surface-exposed ErbB-1 molecules disappeared after a 3 h incubation with EGF, only a 10–25% reduction was induced by MGF or SFGF (Figure 9A). Consistent with the moderate binding affinity of VGF, and the relatively high mitogenic activity of this ligand, it induced only partial downregulation of ErbB-1 (Figure 9A). This pattern of relative receptor downregulation was independent of ligand concentration (Figure 9B). Moreover, a qualitative difference between a viral ligand (SFGF) and the mammalian growth factor was confirmed by performing the following experiment: dose-response assays of ligand-induced receptor phosphorylation were carried out and equipotent concentrations selected (e.g. 62 ng/ml SFGF and 1.2 ng/ml EGF). At these concentrations the difference in potency of receptor downregulation was retained (data not shown), in line with dissimilar mechanisms.

The cellular fate of ErbB-1 after binding of viral ligands was followed by an alternative approach that used immunolocalization and fluorescence microscopy (Figure 9C). In the absence of a ligand, ErbB-1 was localized primarily to the cell surface where it formed either small patches or a uniform pattern. Within 2–5 min after binding of EGF, most ErbB-1 molecules underwent internalization and localized to vesicular structures representing endosomes. Later (15–20 min), these structures assumed a perinuclear localization and their size increased, probably reflecting vesicle fusion and arrival at lysosomal or pre-lysosomal compartments (data not shown). By contrast with an EGF-driven ErbB-1, only limited redistribution of the receptor took place even after long incubation with SFGF (Figure 9C; data not shown). In addition to the

membrane localization of ErbB-1 molecules in SFGF-treated cells, the receptors formed small patches that were either at the cell surface, or very close underneath the plasma membrane. Thus, on the basis of both biochemical and structural lines of evidence it seems that the viral ligand, SFGF, is unable to direct ErbB-1 to large endocytic vesicles and, thereby, to intracellular degradation. Conceivably, the altered routing of ErbB-1 prolongs the active state of the ligand-receptor complex, thus augmenting the mitogenic signal of the viral growth factor.

Discussion

Investigation of animal viruses has provided many insights into basic molecular mechanisms. Perhaps the best example is the lessons in cellular transformation and cell-cycle control that evolved from studies of RNA-containing retroviruses and papovaviruses (e.g. SV-40). Likewise, poxviruses, whose most notable member is the causative agent of smallpox in humans, the variola virus, emerge as a rich source of efficient mechanisms to evade the immune system. For example, the Myxoma T2 protein is a homolog of the tumor necrosis factor- (TNF) receptor that blocks TNF-mediated cytotoxicity (Smith *et al.*, 1990), and vaccinia virus encodes a complement-binding protein that blocks the classical complement pathway (Kotwal and Moss, 1988). Unlike these two proteins that are involved in secondary infection via the efferent lymphatics and blood stream, virus-encoded growth factors play a critical role at the major portal of viral entry, the skin; by induction of localized hyperplasia, additional metabolically active cells become available for viral infection (reviewed in Buller and Palumbo, 1991). However, in contrast to the current notion, our results imply that the viral growth factors do not function simply as alternative agonists of the EGF receptor. Instead, these molecules utilize two novel features: first, each viral ligand is characterized by a unique pattern of specificity to homo- or hetero-dimeric complexes of the ErbB receptors. Secondly, the viral growth factors are biologically far more potent than their mammalian counterparts in terms of receptor occupancy. The underlying molecular mechanisms and their implications for poxvirus pathogenesis are discussed below.

Narrow versus broad ErbB specificity of viral growth factors

Collectively, analyses of cellular proliferation (Figure 2), or survival (Figure 3), ligand displacement (Figure 5), and covalent crosslinking (Figure 6), revealed a ligand-specific pattern of receptor specificity that is schematically presented in Figure 10. This pattern implies that the three viral ligands are functionally distinct. Yet, the mammalian ligand that we used as a reference, namely EGF, differs from the three viral ligands. Thus, whereas EGF can activate all ErbB-1-containing complexes, as well as homodimers of ErbB-4 (when this receptor is overexpressed; Shelly *et al.*, 1998) and ErbB-2/ErbB-4 heterodimers, SFGF differs in that only at very high concentrations (>50 ng/ml) this ligand detectably activates ErbB-4, but, on the other hand, it can efficiently stimulate the ErbB-2/ErbB-3 heterodimer. By contrast, stimulation of the latter by EGF occurs only at very high concentrations (Alimandi

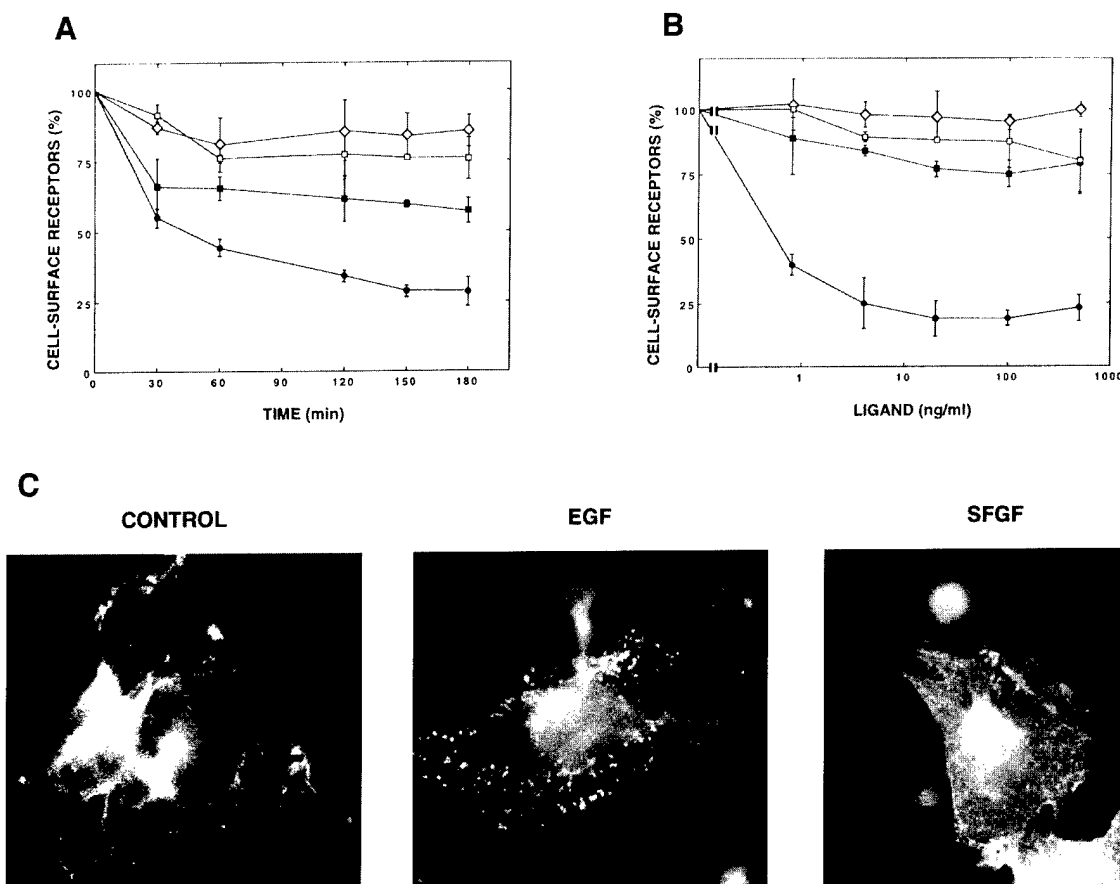


Fig. 9. Intracellular trafficking of ErbB-1 in response to stimulation with SFGF or EGF. CHO cells singly expressing ErbB-1 (denoted CB1 cells) on a low background of the endogenous hamster ErbB-2 were used in all panels. (A and B) Downregulation of ErbB-1 was followed by incubating subconfluent monolayers of CB1 cells (250 000 cells in 24-well trays) with one of the following unlabeled ligands: EGF (●); SFGF (□); VGF (■); and MGF (◇). Cells were treated at 37°C, either for various time intervals with 60 ng/ml ligand (A), or with various ligand concentrations for 2 h (B). Cell surface-bound ligands were removed by acid wash, which was followed by extensive rinsing with binding buffer. The receptor level was then determined by binding of radiolabeled EGF (5 ng/ml) to the cells (2 h at 4°C). (C) For fluorescence labeling of ligand-stimulated ErbB-1, CB1 cells were treated for 5 min at 37°C with EGF or with SFGF, each at 250 ng/ml, as indicated. Control monolayers were treated with buffer alone (CONTROL). Cells were then washed, fixed, permeabilized and stained with antibodies against ErbB-1 (mAb111) as described in Materials and methods. The coverslips were viewed with a Zeiss fluorescence microscope with an oil immersion objective.

et al., 1997; Pinkas-Kramarski *et al.*, 1998). Nevertheless, this property is shared by betacellulin, but not by TGF α , which led to the conclusion that ErbB-1-specific ligands differ in their ability to stimulate the ErbB-2/ErbB-3 heterodimer (Alimandi *et al.*, 1997; Pinkas-Kramarski *et al.*, 1998). This conclusion is in line with the emerging notion (Tzahar and Yarden, 1998) that the multiple EGF-like ligands are distinct in terms of receptor specificity (Beerli and Hynes, 1996; Riese *et al.*, 1996). A remarkable demonstration is provided by MGF; the exclusive receptor of this ligand is the ErbB-2/ErbB-3 heterodimer. It is notable that no other known EGF-like ligand exhibits such a narrow specificity, and neither ErbB-2 nor ErbB-3, when singly expressed, detectably interacts with MGF (data not shown). An intermediate case is provided by VGF: this growth factor binds to and activates cells expressing ErbB-1 in various combinations, but covalent crosslinking analyses of this ligand suggest that it hardly forms heterodimeric complexes of ErbB-1 at all (Figure 6).

Because previous analyses examined the effects of viral ligands on naturally occurring cell lines (e.g. the epidermoid carcinoma A-431 cells) expressing various combinations of ErbBs, it is difficult to compare our

results with previous works. However, it has been reported previously that the cell-binding affinity of MGF is 200-fold lower than that of EGF (Lin *et al.*, 1991), an observation that is consistent with our results (Figure 5). Likewise, synthetic analogs of VGF (Lin *et al.*, 1990) and SFGF (Lin *et al.*, 1988) were found to be 5- to 10-fold less potent than EGF in binding to A-431 cells. Our results with VGF are in line with this observation, but we noted a much higher difference with SFGF (Figure 5). Another discrepancy is related to the biological activity: whereas according to our results SFGF and VGF are mitogenically superior or equivalent to EGF on all ErbB-1-expressing 32D cell derivatives (Figure 2), the previously reported mitogenic effect of SFGF on NRK cells was 10-fold lower than that of EGF (Lin *et al.*, 1988), and VGF was either inactive or antagonistic to EGF (Lin *et al.*, 1990). These differences may, in part, be attributed to the presence of ErbB-4, or to other cell type-specific features. In line with this possibility, we observed an inhibitory effect of VGF on ErbB-1 phosphorylation in epithelial cells, but not in engineered myeloid cells (Figures 1 and 4).

Which selective advantages could poxviruses gain from fine targeting of their growth factors? The mitogenic effect

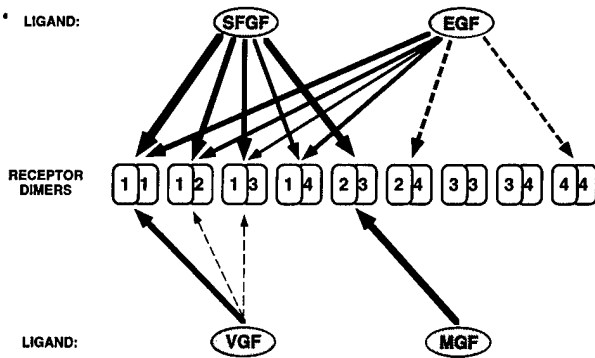


Fig. 10. Receptor specificity of viral growth factors. All possible combinations of ErbB proteins are presented schematically, except for the ErbB-2 homodimer. Specific ErbB proteins are identified by their numbers. The interaction between a growth factor and a specific dimer of ErbBs was inferred on the basis of the following criteria: the ability to stimulate growth of IL-3-dependent cells (Figures 2 and 3); activation of MAPK and receptor phosphorylation (Figure 4); binding to cells expressing specific ErbBs (Figure 5); and covalent crosslinking to specific monomeric and dimeric receptor complexes (Figure 6). These interactions are shown by arrows whose thicknesses represent the relative potency of biological effects (broken arrows indicate weak receptor activation). Interactions that are detectable only at high ligand concentrations (>10 ng/ml) are not represented. For example, the low-affinity interaction between EGF and the ErbB-2/ErbB-3 heterodimer (Alimandi *et al.*, 1997; Pinkas-Kramarski *et al.*, 1998) is not represented. Note that MGF and VGF are narrow-specificity ligands, whereas SFGF and EGF have partially overlapping broad specificities. Nevertheless, wherever the two ligands overlap, SFGF-induced mitogenesis is superior to that of EGF. In addition, productive interaction of EGF with ErbB-4-expressing cells depends on overexpression of this receptor, or on co-expression of ErbB-2.

of the viral growth factors may be restricted to specific lineages of the host's cells. For example, the cells that undergo proliferation upon infection by the Shope fibroma virus (SFV) are undifferentiated fibroblasts, whereas the molluscum contagiosum virus affects primarily epidermal cells. On the other hand, mostly derivatives of a monocyte-macrophage lineage, called histiocytes, are found in the proliferative lesion of the Yaba virus (Sproul *et al.*, 1963). Because ErbB-1 is expressed by most fibroblasts and epithelial cells, but is absent in myeloid cells, whereas ErbB-3 and ErbB-4 are relatively restricted to epithelial cells, receptor specificity may be relevant to virus pathogenesis. Especially relevant is the shared specificity of SFGF and MGF to the ErbB-2/ErbB-3 heterodimeric receptor. This heterodimer is probably the most stable and potent receptor combination of the ErbB family (Pinkas-Kramarski *et al.*, 1996; Tzahar *et al.*, 1996), and its strong proliferative signals presumably underlie the oncogenic effect of an overexpressed ErbB-2 in epithelial tumors (Alimandi *et al.*, 1995; Wallasch *et al.*, 1995). Further, we have previously shown that skin keratinocytes are potently stimulated to proliferate when this heterodimer is induced by NDF (Marikovsky *et al.*, 1995). It is relevant, however, that replacement of the MGF-encoding sequence of the Myxoma virus with that of SFGF, TGF α or VGF resulted in myxomatosis that was clinically and histopathologically indistinguishable from that of the wild-type virus (Opgenorth *et al.*, 1993). Whether or not symptoms related to the fine specificity of each growth factor do not affect pathogenicity, or they simply escaped detection, remains an open question.

Enhancement of signaling by attenuation of receptor inactivation

How does a viral ligand (e.g. SFGF and MGF), whose receptor-binding affinity is significantly lower than that of the mammalian counterpart (e.g. EGF and NDF, respectively), transmit signals that are superior or comparable with that of the physiological ligand? Our results, which are relevant primarily to the SFGF-EGF pair, imply that rather than developing strategies to enhance signal generation, poxviruses evolved mechanisms that attenuate the rapid receptor inactivation process that follows ligand-induced receptor stimulation. In support of this model, ErbB-1 phosphorylation after SFGF binding is relatively low, but it is nevertheless sufficient for full activation of the MAPK (Figure 8A). Moreover, whereas MAPK stimulation by EGF is transient, sustained activation is achieved by SFGF, and the active kinase form is retained in the nucleus for a longer time (Figure 8B). In analogy with the relationships between viral and mammalian ligands, it has been shown repeatedly that transient activation of MAPK in murine fibroblasts results in cell growth, whereas sustained activation leads to cell transformation (reviewed in Marshall, 1995). It is interesting to note that receptor phosphorylation and MAPK activation deviate from precise quantitative coupling (Figure 8). This may reflect signal amplification downstream of the receptor, existence of spare receptors, or the fact that MAPK and ErbB-1 are subject to inactivation by different mechanisms. Besides dephosphorylation, which was not examined by us, receptor downregulation by means of endocytosis is a major process that attenuates growth factor signals (Sorkin and Waters, 1993). This process, however, is very inefficient in the case of the viral ligands (Figure 9). Recent observations made in several signaling systems, including that of ErbB-1 (Galcheva-Gargova *et al.*, 1995), imply that the ubiquitin-proteasome machinery plays a role in directing endocytosed receptors to intracellular degradation (reviewed in Hicke, 1997). This machinery is less efficiently recruited by a viral ligand, SFGF (Figure 8C), and the associated processes of receptor downregulation (Figure 9) and degradation (Figure 8C, lower panel) are severely attenuated. The mechanism underlying attenuation of receptor downregulation is unclear: according to one possibility, tyrosine phosphorylation of ErbB-1, which is conditional for internalization (Glennay *et al.*, 1988) and ubiquitination (Galcheva-Gargova *et al.*, 1995), is insufficiently high in the case of SFGF. Alternatively, although SFGF does not induce formation of large endocytic vesicles containing ErbB-1 (Figure 9C), it is still possible that this ligand, like another potent ErbB-1-specific ligand, namely TGF α (Ebner and Derynck, 1991), targets the receptor to rapid recycling through relatively small endocytic vesicles.

The role of SFGF in cellular hyperplasia near the sites of viral replication is best exemplified by deletion of the SFGF-encoding sequence from the genome of the malignant rabbit fibroma virus (MRV), which resulted in significantly less fatal syndrome and tumors with fewer proliferating cells (Opgenorth *et al.*, 1992). In fact, introduction of DNA of the Shope fibroma virus (SFV) into NIH 3T3 cells transformed these cells in culture, and they became capable of generating tumors in nude mice (Obom and Pogo, 1988). It is therefore conceivable that by

evading signal inactivation SFV potentiated its virulence. Presumably, the extremely low affinity of SFGF to ErbB-1 prevents the rapid endocytic clearance that normally follows growth factor binding to the cell surface, thereby extending the half-life of SFGF at the site of viral infection and replication. Although this mechanism has not been described before with any known EGF-like growth factor, a mutant of EGF (Y13G), engineered for enhanced mitogenic potency, displayed a 50-fold lower binding affinity, slow rate of depletion, and attenuated receptor downregulation (Reddy *et al.*, 1996). Also consistent with the proposed explanation of SFGF potency is the observation that a non-internalizing mutant of ErbB-1 can transform cells once stimulated by EGF (Wells *et al.*, 1990). Likewise, blocking ErbB-1 internalization by mutagenesis of dynamin, a guanosine triphosphatase that is required for clathrin vesicle formation, enhanced EGF-induced mitogenesis (Vieira *et al.*, 1996). We speculate that by adopting a strategy aimed at slow clearance of SFGF and sustained receptor activation, SFV eluded the need for a strong promoter and continuous transcription of the growth factor gene by host cells.

The mechanism underlying the relatively high mitogenic potencies of the other two viral ligands may differ from that of SFGF. The following reasons lead us to propose that VGF, in analogy to TGF α , induces recycling, rather than lysosomal degradation, of its receptor. First, ErbB-1 undergoes only partial downregulation after binding of VGF (Figure 9A). Secondly, receptor recycling is predictable because binding of VGF is relatively sensitive to the low pH characteristic of the maturing endosome, and downregulation of ErbB-1 by VGF is enhanced by monensin, a drug that blocks vesicular transport to the plasma membrane (our unpublished results). The potent action of MGF through the ErbB-2/ErbB-3 heterodimer appears to reflect yet a different mechanism, because the rates of internalization of ErbB-2 and ErbB-3 are much slower than the rate of endocytosis of ErbB-1 (Baulida *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996; Waterman *et al.*, 1998). Clarification of these and other emerging questions that relate to the pathogenesis of poxviruses will require further research. However, in the light of the possibility that all EGF-like ligands carry two receptor binding sites (Tzahar *et al.*, 1997), the data we presented may be useful for the design of ErbB-specific antagonists. In addition, our results underscore the potential physiological importance of low-affinity mammalian ligands, which commonly escape detection, in hyperproliferative and malignant states.

Materials and methods

Materials, buffers and antibodies

EGF (human recombinant) was purchased from Sigma. Epireregulin and a 47 amino acid-long fragment of EGF were synthesized as described (Shelly *et al.*, 1998). A recombinant form of NDF- β 1₁₇₇₋₂₄₆ was from Amgen (Thousand Oaks, CA), and a longer form, HRG β 65 was synthesized as described (Barbacci *et al.*, 1995). Polyclonal rabbit anti-c-Cbl (C-15) antiserum and a monoclonal anti-phosphotyrosine antibody (PY-20) were purchased from Santa-Cruz Biotechnology (Santa-Cruz, CA). Antibodies to ubiquitin were kindly provided by Dr Sadaki Yokota. Anti-Shc antibodies were from USB (Cleveland, OH). mAb111 and mAb199 directed to the extracellular domain of ErbB-1 were generated in our laboratory essentially as described (Chen *et al.*, 1996). A polyclonal anti-ErbB-1 antibody (anti-C-terminal) was raised against a 14 amino

acid-long peptide that corresponded to the C-terminal sequence of the ErbB-1. For detection of activated MAP-kinase we used a mAb directed to the doubly phosphorylated form of MAP-kinase in which both tyrosine and threonine residues of the TEY motif were phosphorylated (Yung *et al.*, 1997). Binding buffer contained RPMI medium with 0.2% bovine serum albumin (BSA). HNTG buffer contained 20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol. Solubilization buffer contained 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1.5 mM EGTA, 2 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin and leupeptin, each at 10 μ g/ml.

Synthesis of viral peptides

Peptides corresponding to the EGF-like motif of the three viral proteins were synthesized on an Applied Biosystems (ABI) model 431 peptide synthesizer fortified with UV feedback monitoring at 301 nm, and using FMOC-Rink amide AM resin. The conventional ABI monitor-previous-peak-algorithm was employed up to five times with a cut-off of 3.5% of the first deprotection. A secondary deprotection [using 2% DPU/2% piperidine/96% *N*-methylpyrrolidone (NMP)] was performed and followed by double coupling. Acetic anhydride/hydroxybenzotriazole (HOBT) capping was utilized at the end of each coupling, followed by washing with 1:1 trifluoroethanol/dichloromethane (DCM). The peptides were deprotected and removed from the resin as described (King *et al.*, 1990), with the following modifications: methoxyindole (2%) was added to reagent K, and the reaction time was changed to 3.5 h. Small quantities of the reduced peptides were purified by reverse-phase high performance liquid chromatography and examined by matrix-assisted laser desorption ionization (MALDI) mass spectral analysis. The crude reduced proteins were dissolved in a Tris-HCl buffer pH 6.0 containing 6 M guanidium-HCl and diluted to a concentration of 0.06 mg/ml in methionine-containing buffer (10 mM) that included 1.5 mM cysteine, 0.75 mM cysteine, and 100 mM Tris pH 8.0. The mixture was stirred for 48 h cold, and the oxidized peptide isolated on a C-4 VYDAC 10 micron preparative column (22 \times 250 mm) using a 0.1% trifluoroacetic acid-water/acetonitrile gradient. EGF₁₋₄₇ was similarly synthesized, starting at the seventh residue of the 53 amino acid-long EGF sequence. The oxidized proteins were lyophilized and characterized by mass spectrometry and amino acid analysis, and shown to be homogeneous. Electro-spray mass spectrometry was used to verify the mass of the synthetic peptides. Disulfide bonding patterns were analyzed by using a mixture of proteolytic enzymes (trypsin and Glu-C) and MALDI mass spectrometry. The canonical pattern of the EGF-like motif (Cys1-Cys3, Cys2-Cys4 and Cys5-Cys6) was confirmed.

Cell lines and tissue culture

Derivatives of the 32D murine hematopoietic progenitor cell line were grown in RPMI-1640 medium supplemented with antibiotics, 10% heat-inactivated fetal bovine serum (FBS), and 0.1% medium that was supplied with IL-3. The various sublines expressing ErbB combinations were described previously (Pinkas-Kramarski *et al.*, 1996), except for the ErbB-4-expressing line (D4), whose level of expression was ~10-fold higher than in D24 and in D34 cells, due to the use of different promoters (denoted E4 cell line in Alimandi *et al.*, 1997). Chinese hamster ovary (CHO) cells expressing combinations of ErbB proteins were previously described (Tzahar *et al.*, 1996). These cells were grown in DMEM/F12 (1:1) medium supplemented with antibiotics and 10% heat-inactivated FBS.

Cell proliferation assays

Cells were washed free of IL-3, resuspended in RPMI-1640 medium supplemented with calf serum (10%) at 5×10^5 cells/ml, and treated without or with growth factors or IL-3 (1:1000 dilution of medium conditioned by IL-3-producing cells). Cell survival was determined by using the [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl] tetrazolium bromide (MTT) assay as previously described (Pinkas-Kramarski *et al.*, 1996). MTT (0.1 mg/ml) was incubated for 2 h at 37°C with the analyzed cells. Living cells can transform the tetrazolium ring into dark blue formazan crystals, that can be quantified by optical density at 540–630 nm after lysis of the cells with acidic isopropanol (Mosman, 1983). For dose-response analyses, serial dilutions of the ligands were added in RPMI-1640 medium, and cells were harvested 24 h after plating. For cell survival experiments, cells were treated without or with various ligands (100 ng/ml) or IL-3, and survival determined 24, 48 or 72 h later.

Ligand binding and covalent crosslinking analyses

Growth factors were labeled using Iodogen as described (Karunakaran *et al.*, 1995). The specific activity ranged between 0.5 and 5×10^5 c.p.m./ng.

Ligand displacement analyses with 32D cells were performed as described (Pinkas-Kramarski *et al.*, 1996) with the following modifications: cells were washed once with binding buffer, divided into tubes (final volume 0.1 ml) and then incubated for 2 h at 4°C with the radiolabeled EGF, or NDF, and various concentrations of unlabeled ligands. Nonspecific binding was determined in the presence of the corresponding unlabeled ligand at 1 µg/ml. Ligand binding to CHO cells was performed as described (Tzahar *et al.*, 1996). Chemical crosslinking experiments of the viral peptides were performed as follows: the various 32D cell lines (1×10^7 cells) were incubated for 2 h on ice with the radiolabeled ligand (250 ng/ml). The chemical crosslinking reagent bis(sulfosuccinimidyl) substrate (BS³) was then added to 1 mM final concentration. After 45 min at 4°C cells were washed with phosphate-buffered saline (PBS). Cell lysates were prepared and analyzed by immunoprecipitation using mAbs against the four ErbB proteins, or control mAbs, as described (Tzahar *et al.*, 1996). This assay was also performed in the presence of mAbs against ErbB-1, either mAb111 or mAb199, each at 20 µg/ml. In this case, the immunoprecipitation step was performed using a polyclonal rabbit antibody to ErbB-1.

Lysate preparation and immunoprecipitation

Cells were exposed to the indicated stimuli in RPMI-1640 medium or in binding buffer. After treatment, cells were collected by centrifugation and solubilized in lysis buffer. Lysates were cleared by centrifugation. For direct electrophoretic analysis, boiling gel sample buffer was added directly to the cells and the lysates were mixed vigorously to break genomic DNA. For crosslinking experiments, lysates were first subjected to immunoprecipitation with various antibodies. Rabbit antibodies were directly coupled to protein A-Sepharose while shaking for 1 h at 4°C. Mouse antibodies were first coupled to rabbit-anti-mouse immunoglobulin G and then to protein A-Sepharose by using the same procedure. The proteins in the lysate supernatants were immunoprecipitated with aliquots of the protein A-Sepharose-antibody complex for 2 h at 4°C. The immunoprecipitates were washed three times with HNTG, and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Either 6% acrylamide gels (crosslinking assays) or 8.5% acrylamide gels (receptor phosphorylation and MAPK assays) were used. Proteins were then electrophoretically transferred to nitrocellulose membranes that were blocked for 2 h in TBST buffer (0.02 Tris-HCl pH 7.5, 0.15 M NaCl and 0.05% Tween 20) containing 5% milk, and blotted with primary antibodies for 2 h. This was followed by a secondary antibody linked to horseradish peroxidase. For receptor phosphorylation and MAPK activation experiments, the upper part of the membrane was used for immunoblotting with an anti-phosphotyrosine mAb, and the lower part of the membrane was used for immunoblotting with a mAb to the active form of MAPK. Immunoreactive bands were detected with an enhanced chemiluminescence reagent (Amersham Corp).

Receptor downregulation assays

CB1 cells grown in 24 well plates were washed with binding buffer and then incubated at 37°C for up to 3 h with various ligands to allow receptor downregulation. Cells were then put on ice, rinsed twice with binding buffer and surface-bound ligand removed by acid wash (0.5 ml solution of 150 mM acetic acid at pH 2.7, containing 150 mM NaCl). The receptor level was then determined by binding of radiolabeled EGF (5 ng/ml) to the cells for 2 h at 4°C. Cell-associated radioactivity was determined after collecting a second acid wash.

Immunofluorescence

CB1 cells were plated on glass coverslips and grown for 24 h. Cells were rinsed with serum-free medium and then treated with a growth factor (250 ng/ml) for 5 min at 37°C. Treated coverslips were fixed with 3% paraformaldehyde in PBS (15 min), and then rinsed with PBS. Cells were permeabilized for 10 min at 22°C with PBS containing 1% BSA and 0.2% Triton X-100. This was followed by a 10 min incubation with PBS. For immunodetection of ErbB-1, coverslips were incubated for 1 h at 22°C with mAb111. After extensive washing with PBS, the coverslips were incubated with a Cy3-conjugated goat-anti-mouse [F(ab')₂ specific] antibody (Jackson ImmunoResearch Laboratories) and washed three times with PBS. Coverslips were viewed with a Zeiss fluorescence microscope in oil immersion. For immunodetection of activated MAPK we used D1 cells. After stimulation with ligands (at 50 ng/ml) cells were washed with PBS, spun onto a glass slide, and fixed in 3% formaldehyde in PBS solution for 30 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, and reacted with a mAb directed to the active form of MAPK (Yung *et al.*, 1997). Following extensive washing with PBS, antibody detection was performed using rhodamine-conjugated goat anti-mouse IgG (1:1000, Jackson Immuno-

Research Laboratories). To visualize nuclear DNA, cells were stained with 4,6-diamidino-2-phenylindole (DAPI, dissolved in PBS).

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Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase

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The ErbB/HER family of receptor tyrosine kinases consists of four receptors that bind a large number of growth factor ligands sharing an epidermal growth factor- (EGF)-like motif. Whereas ErbB-1 binds seven different ligands whose prototype is EGF, the three families of neuregulins (NRGs) activate ErbB-3 and/or ErbB-4. Here we characterize a fourth neuregulin, NRG-4, that acts through ErbB-4. The predicted pro-NRG-4 is a transmembrane protein carrying a unique EGF-like motif and a short cytoplasmic domain. A synthetic peptide encompassing the full-length EGF-like domain can induce growth of interleukin-dependent cells ectopically expressing ErbB-4, but not cells expressing the other three ErbB proteins or their combinations. Consistent with specificity to ErbB-4, NRG-4 can displace an ErbB-4-bound NRG-1 and can activate signaling downstream of this receptor. Expression of NRG-4 mRNA was detected in the adult pancreas and weakly in muscle; other tissues displayed no detectable NRG-4 mRNA. The primary structure and the pattern of expression of NRG-4, together with the strict specificity of this growth factor to ErbB-4, suggest a physiological role distinct from that of the known ErbB ligands.

Keywords: growth factor; oncogene; pancreas; signal transduction; tyrosine kinase

Introduction

Cell-to-cell signaling is an essential feature of multicellular organisms, playing important roles in both the unfolding of developmental diversification as well as mediating the homeostasis of vastly different cell types. A large number of tyrosine kinase growth factor receptors play key roles in this process. Type-1 tyrosine kinase receptors, also known as ErbB/HER proteins, comprise one of the better-characterized families of growth factor receptors, of which the epidermal growth factor receptor (ErbB-1) is the prototype (reviewed in Burden and Yarden, 1997). The ErbB family constitutes four known receptors which dimerize upon ligand stimulation, transducing their signals by subsequent autophosphorylation catalyzed by an intrinsic cytoplasmic tyrosine kinase, and recruiting downstream signaling cascades.

The ErbBs are activated by a large number of ligands. Depending upon the activating ligand, most

homodimeric and heterodimeric ErbB combinations can be stabilized upon ligand binding (Tzahar *et al.*, 1996), thus allowing a complex, diverse downstream signaling network to arise from these four receptors. The choice of dimerization partners for the different ErbBs, however, is not arbitrary. Spatial and temporal expression of the different ErbBs do not always overlap *in vivo*, thus narrowing the spectrum of possible receptor combinations that an expressed ligand can activate for a given cell type (Erickson *et al.*, 1997; Gassmann *et al.*, 1995; Lee *et al.*, 1995; Pinkas-Kramarski *et al.*, 1997; Riethmacher *et al.*, 1997). Furthermore, a hierarchical preference for signaling through different ErbB receptor complexes takes place in a ligand-dependant manner. Of these, ErbB-2-containing combinations are often the most potent, exerting prolonged signaling through a number of ligands, likely due to an ErbB-2-mediated deceleration of ligand dissociation (Karunagaran *et al.*, 1996; Tzahar *et al.*, 1996; Wang *et al.*, 1998). In contrast to possible homodimer formation of ErbB-1 and ErbB-4, for ErbB-2, which has no known direct ligand, and for ErbB-3, which lacks an intrinsic tyrosine kinase activity (Guy *et al.*, 1994), homodimers either do not form or are inactive. Heterodimeric ErbB complexes are arguably of importance *in vivo*. For example, mice defective in genes encoding either NRG-1, or the receptors ErbB-2 or ErbB-4, all result in identical failure of trabeculae formation in the embryonic heart, consistent with the notion that trabeculation requires activation of ErbB-2/ErbB-4 heterodimers by NRG-1 (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995).

At the biochemical level, the known ErbB ligands fall into several categories (Riese *et al.*, 1996b). One category, the ErbB-1 ligands, includes EGF, transforming growth factor α (TGF α), epiregulin, amphiregulin, betacellulin and the heparin-binding EGF (HB-EGF) (Higashiyama *et al.*, 1991; Marquardt *et al.*, 1984; Shing *et al.*, 1993; Shoyab *et al.*, 1989; Toyoda *et al.*, 1995). To different extents, these ErbB-1 binding ligands can also activate other receptors of the ErbB family, and hence may mediate distinct signaling outputs for a given cell type (reviewed in Tzahar and Yarden, 1998). Another category of ErbB ligands consists of the Neuregulin (NRG) family. NRG-1 (also named Neu differentiation factor (NDF), heregulin, glial growth factor, and acetylcholine receptor inducing activity) was first identified by its ability to indirectly phosphorylate ErbB-2 (Holmes *et al.*, 1992; Peles *et al.*, 1992; Wen *et al.*, 1992). Subsequently, NRG-1 was found to directly bind ErbB-3 and ErbB-4 and to sequester ErbB-2 by receptor dimerization (Peles *et al.*, 1993; Plowman *et al.*, 1993; Sliwkowski

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et al., 1994; Tzahar *et al.*, 1994). Multiple isoforms of NRG-1 exist which amongst other roles, permit heterogeneous binding affinities to different ErbB complexes (Tzahar *et al.*, 1994). The NRG family now includes also two isoforms of NRG-2 (Busfield *et al.*, 1997; Carraway *et al.*, 1997; Chang *et al.*, 1997; Higashiyama *et al.*, 1997), of which the alpha isoform is a pan-ErbB ligand (Pinkas-Kramarski *et al.*, 1998), and NRG-3, a ligand of ErbB-4 (Zhang *et al.*, 1997). The multiplicity of genes encoding ErbB-1 ligands, contrasting with the small number of known genes encoding ligands for ErbB-3 or ErbB-4 (namely: NRGs), led us to believe in the existence of additional NRG genes in the genome of mammals. Here we report on a fourth neuregulin, denoted NRG-4, which acts through the ErbB-4 receptor tyrosine kinase. In addition to its novel structure, this growth factor displays a pattern of expression different from other EGF-like molecules.

Results

Identification of a candidate novel ErbB ligand

With the assumption that there may still exist novel ErbB-specific ligands we decided to search for new family members by homology. The recent explosion of DNA sequencing data added to DNA databases, largely resultant from the Human Genome Project initiative, offers scanning of these data for novel transcripts coding ligands with homology to the ErbB-3- and ErbB-4-specific ligand, NRG-1 (NDF). The motif CX₂CXNGGX₂CX₂CX₂YXGXRC, conserved in most isoforms of NRG-1, was used to scan available new DNA sequences. An expressed sequence tag (EST) clone originating from a mouse liver cDNA library (accession number AA238077) was identified, its sequence encoding an EGF-like domain sharing 32% identity with the NRG-1 β isoform (Wen *et al.*, 1992). This clone was obtained and fully sequenced, its presumed translation product encoding a protein of 115 amino acids (Figure 1a). Hydropathy analysis using the Kyte-Doolittle algorithm (Kyte and Doolittle, 1982) supports the existence of a transmembrane domain (Figure 1b) characteristic to most NRG isoforms (Marchionni *et al.*, 1993; Wen *et al.*, 1994). Conspicuously, this protein sequence lacks a hydrophobic amino-terminal stretch, commonly found in signal peptide motifs, important in sequestering proteins to traverse the plasma membrane. Most isoforms of NRG-1 also lack consensus signal peptide sequences, but they carry an apolar N-terminal sequence thought to allow transmembrane orientation of the precursor molecule. The predicted extracellular domain of the precursor protein includes the EGF-like domain, whose primary structure displays the entire structural motifs characteristic to the EGF/NGR family (Figure 1c). The putative cytoplasmic domain of the precursor protein is relatively short and contains one potential site for N-glycosylation. Two additional sites are located at the probable ectodomain.

Alignment of the EGF-like domains of all known ErbB-specific ligands of mammalian origin indicated that the novel transcript encodes a new member of this

family (Figure 1c). Its characteristic six extracellular cysteine residues and their conserved spacing predict the existence of the three disulfide bridges, denoted as A, B and C, that are the landmark of all EGF-like peptides. Besides the six conserved cysteine residues, the new EGF-like domain shares very high homology with other members of the NRG family, including a glycine at position 21 (Gly-21), Gly-42 and Arg-44, along with many semi-conserved residues. Of note, the expected B loop of the new protein, like the loops of EGF and NRG-2, is shorter by three residues. Except for the EGF-like domain and the transmembrane topology of the novel predicted protein, it shares no

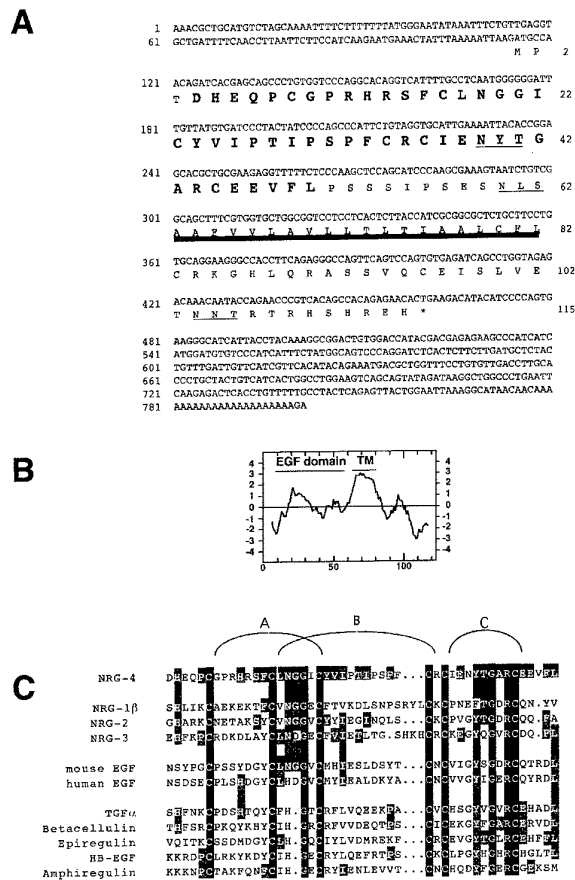


Figure 1 The primary structure of NRG-4. (a) Nucleotide sequence and deduced amino acid sequence of the predicted pro-NRG-4 transcript. Nucleotides are numbered at the left-hand column and amino acids at the right-hand column. The EGF-like domain with its six cysteine residues is shown in bold type, and potential N-glycosylation sites are underlined. The filled box underlines the predicted transmembrane amino acid sequence. (b) Hydropathy profile of pro-NRG-4. The method of Kyte and Doolittle (1982) was used with a window of 11 residues. Positive values indicate increasing hydrophobicity. Amino acid numbers are indicated below the profile. The putative transmembrane stretch of the pro-NRG-4 is marked. Note the absence of a recognizable signal peptide at the N-terminus. (c) Alignment of the amino acid sequence of the EGF-like domain of NRG-4 with the EGF-like motifs of other growth factors. Canonical residues are boxed in black. Other identities with NRG-4 are shaded in gray. The predicted three disulfide bonds of the motifs (Cys 1-3, Cys 2-4 and Cys 5-6) are shown above the alignment and labeled as loops A, B and C. The abbreviations used are as follows: NRG, neuregulin; TGF α , transforming growth factor α ; HB-EGF, heparin-binding EGF-like growth factor. If not specified, the species of origin of all ligands is murine, except NRG-1 (rat)

significant sequence homology or structural motifs with other ErbB ligands.

Tissue-specific expression of the novel transcript

Northern blot analysis of mRNA isolated from different human adult tissues revealed moderate expression of the novel transcript in skeletal muscle and high levels in the pancreas (Figure 2). Other tissues, including brain and placenta, two rich sources of many different growth factors, displayed very low, if any, expression. Three discernible molecular weight species (0.8, 1.8 and 3.0 kilobases) were detectable in pancreas and in muscle, indicating the existence of several mRNA isoforms, the smallest band consistent in size with the clone described in this study.

The EGF-like domain of NRG-4 stimulates proliferation of ErbB-4-expressing cells

To test the prediction that the novel transcript encodes an ErbB-specific ligand, we synthesized the corresponding full-length EGF-like domain (residues 4–50, Figure 1a), denatured and refolded the synthetic peptide to allow proper disulfide bridging. This method has been used before to synthesize functionally active derivatives of other EGF-like growth factors (Barbacci *et al.*, 1995; Lin *et al.*, 1988; Shelly *et al.*, 1998). Previously we have established a series of derivatives of the 32D cell line engineered to express different ErbB receptors or their combinations (Pinkas-Kramarski *et al.*, 1996; Shelly *et al.*, 1998). The myeloid 32D parental cells require cytokine stimulation, such as interleukin 3 (IL3) for their growth, and were chosen because they lack endogenous ErbB expression. Signaling through different ErbB-receptors can replace the IL3-dependent mitogenicity and survival for these cell lines, and hence this system provides a sensitive means to detect ligand-induced growth signals, which are conveniently

measured as a function of cellular metabolic activity by using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl] tetrazolium bromide (MTT) assay (Mosman, 1983).

Cells singly expressing ErbB-1, ErbB-2 or ErbB-3 (denoted D1, D2 or D3, respectively) did not respond to the synthetic novel peptide in a 24-h dose-response assay, although responses to EGF (D1 cells), an ErbB-2-stimulatory monoclonal antibody (D2 cells, (Klapper *et al.*, 1997)), or IL-3 (D3 cells) were retained (Figure 3a, and data not shown). The latter cell line is not responsive to NRGs due to the defective kinase of ErbB-3. However, ErbB-4 expressing cells (D4), exhibited a modest dose-dependent mitogenic response in comparison to its counterpart NRG-1 β control. Because different heterodimeric complexes of ErbB proteins can diversify and enhance signaling by EGF-like ligands (Cohen *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996; Riese *et al.*, 1995), cells co-expressing two ErbB proteins (for example D12 cells co-express ErbB-1 and ErbB-2) were also tested for NRG-4-induced mitogenicity. Of the tested combinations, namely: D12, D13, D23 and D24 cells, a cell line expressing a combination of ErbB-4 with ErbB-2 (D24 cells) was the only line that responded mitogenically to the novel peptide (Figure 3a). Notably, co-overexpression of ErbB-1 and ErbB-2 resulted in a relatively high basal proliferation activity, but these cells still responded to EGF (Figure 3b, and data not shown). Additionally, in cells co-overexpressing ErbB-2 and ErbB-4, NRG-1 and the novel ligand were almost equipotent (compare D4 and D24 panels in Figure 3a), indicating that ErbB-2 can enhance the mitogenic effect of the novel ligand, as it does for other ErbB ligands (Graus-Porta *et al.*, 1995; Karunakaran *et al.*, 1996; Wang *et al.*, 1998).

A long-term cell survival assay confirmed the ability of the novel growth factor to stimulate ErbB-4. This assay examined the ability of added growth factors to sustain survival of certain 32D derivatives in the absence of IL-3. As in the dose-response experiments, the novel synthetic peptide only stimulated the survival of the two ErbB-4-expressing cell lines we examined, namely D4 and D24 cells (Figure 3b). Also similar to the short-term dose response assay, stimulation of D24 cells was more robust, and akin to the NRG-1-treated controls than was the response of D4 cells. These data indicate that the novel growth factor can exert a weak proliferative signal through ErbB-4 alone, but co-expression of ErbB-2 with ErbB-4 allows a superior mitogenic response, as it does in the case of NRG-1 (Wang *et al.*, 1998). On the basis of the ability of the novel synthetic peptide to mediate a biological effect through one of the neuregulin receptors we named it neuregulin-4 (NRG-4).

NRG-4 recognizes and activates ErbB-4

To elucidate the molecular interactions pertaining to NRG-4 signaling, several different approaches were employed to test specific binding of this growth factor to the four ErbB proteins. In the first assay, binding studies in a cell-free system were performed with recombinant soluble forms of all four ErbB proteins. The soluble proteins, denoted IgB-1 through -4, consist of a dimeric fusion between the extracellular domain of the corresponding ErbB and the Fc portion of a

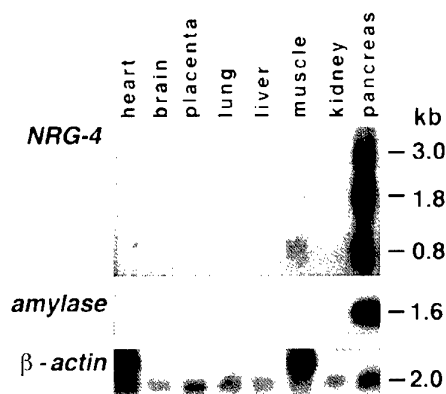


Figure 2 Northern blot analysis of NRG-4 expression in human tissues. Poly(A)-containing RNA from the indicated human tissues (2 μ g per lane) was analysed using a nitrocellulose filter purchased from Clontech (San Diego, CA, USA). The blot was hybridized with a full-length NRG-4 cDNA probe radiolabeled using the Klenow fragment of DNA polymerase I and random hexamers as primers. Following autoradiography, the filter was stripped of radioactivity and re-probed sequentially with pancreas and muscle markers, *alpha-amylase-2* and *beta-actin*, respectively. Molecular weights of marker molecules are indicated in kilobases (kb). Note that *beta-actin* probe also hybridized with a larger molecular weight isoform present in heart and in skeletal muscle

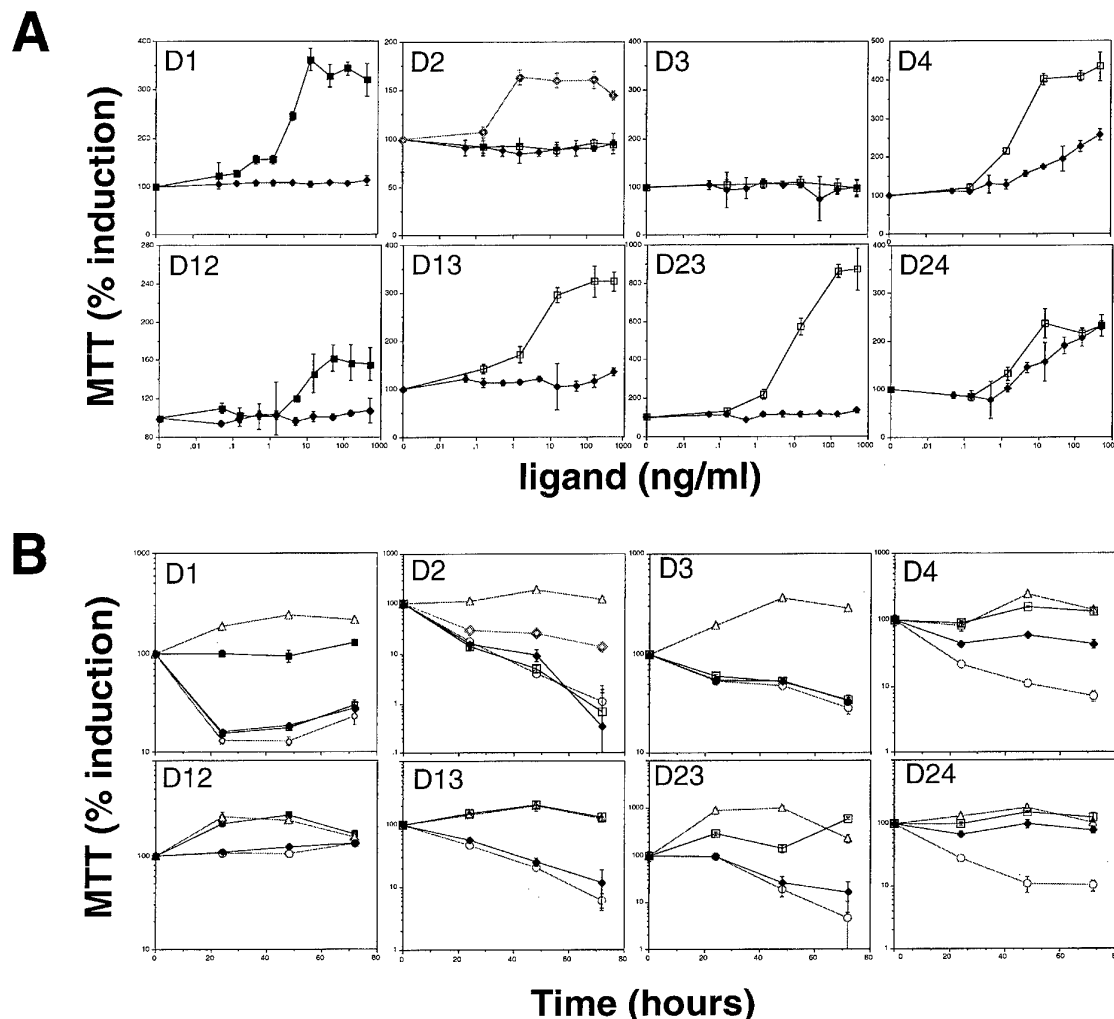


Figure 3 Proliferative and survival effects of NRG-4 on ErbB-expressing derivatives of 32D cells. (a) Cell proliferation assay. The indicated derivative lines of 32D cells were tested for cell proliferation using the MTT assay. Cells were deprived of IL-3 and then plated at a density of 5×10^5 cells/ml in media containing serial dilutions of NRG-4 (closed diamonds), EGF (closed squares), NRG-1 β (open squares), or the L96 (maximal dose: 50 μ g/ml) anti-ErbB-2 monoclonal antibody (open diamonds). The MTT assay was performed 24 h later. Results are presented as per cent induction over the control untreated cells, and are the mean \pm s.d. of four determinations. Each experiment was repeated at least twice with similar findings. Note that no responses to EGF-like ligands were observed with cells expressing either ErbB-2 or ErbB-3 alone, but these cell derivatives retained response to IL-3 (data not shown). (b) Cell survival assay. The indicated derivatives of 32D cells were incubated for various time intervals in the absence of IL-3. The following ligands, each at a concentration of 100 ng/ml, were incubated with cells: NRG-4, EGF, NRG-1 β , or 50 μ g/ml mAb L96 (symbols are as described in (a)). For control, cells were incubated with medium conditioned by IL-3-producing cells (open triangles), or with no factor (open circles). The extent of cell proliferation was determined daily by using the colorimetric MTT assay. The data presented are the mean \pm s.d. of four determinations. Note that co-expression of ErbB-1 and ErbB-2 (D12 cells) enabled cell survival in the absence of IL-3. The experiment was repeated twice with similar results.

human immunoglobulin G (Chen *et al.*, 1996). NRG-4, EGF and NRG-1 β were radiolabeled with 125 I, incubated with the soluble receptors, and then irreversibly bound to the IgBs using the BS³ covalent crosslinking reagent. As expected for the controls, a strong signal was detected for EGF binding to IgB-1 in contrast to NRG-1 β , which bound strongly to IgB-3 and IgB-4, but no ligand bound to IgB-2 (Figure 4a). In comparison to NRG-1, 125 I-NRG-4 bound to the soluble form of ErbB-4 (IgB-4) only weakly, with low or no binding to the other IgB proteins (Figure 4a). To confirm specificity of the covalent crosslinking assay we co-incubated unlabeled NRG-4, at 100-fold molar excess, together with the radioactive ligand and

observed efficient displacement from IgB-4 (lower panel of Figure 4a). Thus, consistent with the ability of NRG-4 to induce growth and survival of ErbB-4-expressing cells, but not cells singly expressing the other three ErbBs, this ligand recognized only ErbB-4 (IgB4) in solution.

To test the prediction that NRG-4 can recognize a surface-expressed ErbB-4, but no other membrane-bound ErbB protein, we used a Chinese hamster ovary (CHO) cell line. These cells express low amounts of ErbB-2, but no other ErbB receptor, and accordingly did not bind NRG-4 or any other neuregulin (Tzahar *et al.* (1996), and data not shown). CHO cells were transfected with plasmid vectors directing expression of

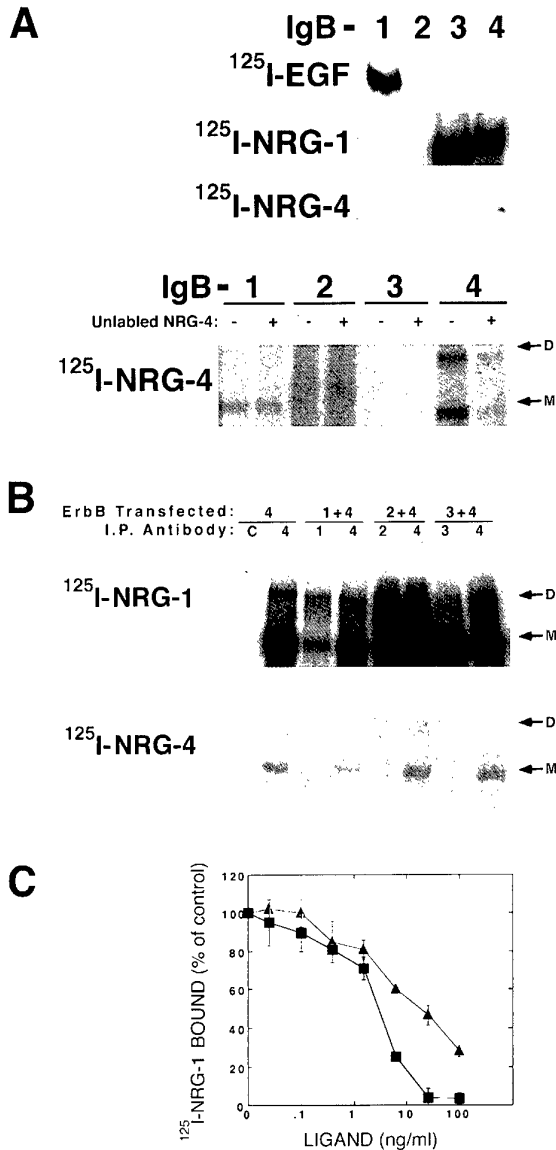


Figure 4 NRG-4 binding to ErbB proteins. (a) Covalent crosslinking of NRG-4 to recombinant-soluble ErbB proteins. The four soluble forms of ErbB proteins, in the form of IgG fusions (denoted IgB-1 through -4), were separately incubated with the indicated radiolabeled growth factors. Where indicated (lower panel), an excess (100-fold) of unlabeled NRG-4 was co-incubated with the labeled ligand. Following 2 h at 22°C, the covalent crosslinking reagent BS³ was added (1 mM) and 45 min later the ligand-receptor complexes were immunoprecipitated with agarose-immobilized protein-A beads. Arrows mark the locations of monomeric (M) and dimeric (D) receptor complexes. (b) Covalent crosslinking of NRG-4 to cell surface-expressed ErbB proteins. CHO cells were transfected with vectors directing expression of the indicated ErbB proteins or their combinations. Two days later cell monolayers were incubated with either ¹²⁵I-NRG-1β or ¹²⁵I-NRG-4 (EGF-like domains, each at 100 ng/ml). Following 2 h at 4°C, the covalent crosslinking reagent bis(sulfosuccinimidyl)-suberate (BS³) was added (1 mM final concentration) and cell extracts prepared after an additional 45 min of incubation. The indicated ErbB proteins were then immunoprecipitated (I.P.) with mouse monoclonal antibodies, and the complexes resolved by gel electrophoresis and autoradiography. Arrows mark the locations of monomeric (M) and dimeric (D) receptor complexes. (c) Binding of NRG-4 to ErbB-4-expressing cells. Displacement analyses of radiolabeled NRG-1β were performed with CHO cells expressing ErbB-4. Cell monolayers (2 × 10⁵ cells) were incubated for 2 h at 4°C with a radiolabeled NRG-1 (5 ng/ml) in the presence of increasing

ErbB-4, or co-transfected with an ErbB-4 plasmid together with vectors expressing one of the three other ErbB proteins. Two days later, cells were incubated with ¹²⁵I-NRG-4, or with a radiolabeled NRG-1 as control, and subsequently the formed ligand-receptor complexes were stabilized by using a covalent cross-linking reagent. Immunoprecipitation of the expressed ErbB proteins allowed analysis of the covalently held complexes. Expression of ErbB-4 alone conferred to CHO cells the ability to form complexes with NRG-4, as well as with NRG-1 (Figure 4b, and data not shown). In line with the lower mitogenic activity of NRG-4, the covalent crosslinking signal obtained with this ligand was weaker than that observed with a radioactive NRG-1. Nevertheless, both monomers and dimers of ErbB-4 were formed by the two ligands (detection of NRG-4-containing dimers required long film exposures). Co-expression of ErbB-1 or ErbB-3 did not significantly affect the radioactive signals, but in the case of ErbB-2 an enhancement effect was observed with NRG-1. The ability of anti-ErbB-1 and anti-ErbB-2 antibodies to precipitate NRG-4-labeled monomeric and dimeric receptor species (Figure 4b) is probably due to co-immunoprecipitation of ErbB-4 and it indicates the existence of NRG-4-promoted heterodimers with ErbB-1 and ErbB-2. Interestingly, ErbB-3 largely escaped heterodimerization with ErbB-4 following binding of NRG-1 or NRG-4.

Taken together, the biological effects of NRG-4 and its complex formation with ErbB-4 implied not only specificity of recognition, but also weaker interaction relative to NRG-1. To quantify the interaction, we performed ligand displacement analysis on ErbB-4-expressing CHO cells. The ability of unlabeled NRG-4 to displace surface-bound radiolabeled NRG-1β was compared with that of unlabeled NRG-1. The results of this experiment indicated an approximately eightfold lower binding affinity of NRG-4 to ErbB-4 (Figure 4c). While NRG-1 bound with an apparent affinity that lies in the low nM range, NRG-4 displayed an apparent approximate K_d of 20 nM. In conclusion, NRG-4 specifically binds to ErbB-4 with an affinity that is lower than that of NRG-1β. Because we previously reported that relative to NRG-1β the alpha isoform displays a 5–8-fold lower affinity to both ErbB-3 and ErbB-4 (Tzabar *et al.*, 1994), it is conceivable that NRG-4 and NRG-1α bind to ErbB-4 with similar affinities.

Evidently, NRG-4 binds to ErbB-4 and mediates cell proliferation through activation of this receptor. Because other ErbB ligands stimulate cell growth via tyrosine phosphorylation of their respective receptors and activation of the intervening mitogen-activated protein kinase (MAPK) cascade, we tested these two signaling steps in NRG-4-responsive myeloid cells expressing ErbB-4 (D4 and D24 cell lines). Cells were stimulated with 100 ng/ml of activating ligand for 5 min, followed by lysis and analysis by immunoblotting. NRG-4 stimulated phosphorylation of the 180-kDa ErbB receptors in D4 and in D24 cells with an

concentrations of an unlabeled NRG-4 (closed triangles), or NRG-1β (closed squares). Each data point represents the mean and range (bars) of two determinations

accompanying activation of MAP-kinase (Erk-1 and Erk-2) also detected (Figure 5). In contrast to these two cell lines, and consistent with the growth and binding assays, NRG-4 at doses as high as 1 μ g/ml, did not stimulate the other 32D cell lines (D1, D2, D3, D12, D13 and D23 cells, data not shown). These results further support the conclusion that NRG-4 is a bona fide ligand of the ErbB receptor family that selectively interacts with receptor complexes containing ErbB-4.

Discussion

In this paper we identify and present the initial characterization of NRG-4, a new cognate ligand of the EGF/NGF family. Aside from NRG-4 possessing a neuregulin-like EGF domain (Figure 1c), it shares very little other sequence homology to the known NRGs, particularly in the vicinity of the transmembrane domain, a region where the other three NRGs exhibit high primary sequence homology. However, the presumed precursor form of NRG-4 shares several structural characteristics with other mammalian ErbB ligands (reviewed in Massague and Pandiella (1993)), including a transmembrane topology, a juxtamembrane location of the EGF-like domain, and a putative proteolytic cleavage site located at a serine-rich region C-terminally to the EGF-like domain. This region may serve as a site of O-glycosylation, in addition to two potential sites of N-glycosylation located in the presumed ectodomain of NRG-4. Like other NRGs, but unlike most ErbB-1-specific ligands, NRG-4 lacks an N-terminally located hydrophobic signal peptide. However, the absence of a characteristic sequence may not exclude the possibility that NRG-4 acts as a secreted growth factor, because other signal peptide-less growth factors can be secreted or released from producer cells through alternative secretory mechanisms or upon cell lysis. NRG-4 presents a rather unique case as it also lacks an apolar stretch of amino acids that usually replaces a signal peptide (e.g., in NRG-1).

In fact, the presumed ectodomain of NRG-4 is the shortest among NRG/EGF family members. In addition, unlike other NRGs, which contain a variety of structural motifs, such as an immunoglobulin-like domain, a cysteine-rich region, or a mucin-like domain, NRG-4 contains no recognizable structural motif other than the EGF-like domain.

That the EGF-like domain of NRG-4 functions as a receptor-binding moiety is indicated by our *in vitro* studies with engineered cell lines and also with breast cancer cells naturally expressing all four ErbB proteins (Figures 3–5, and data not shown). The EGF-like domain of NRG-4 exhibits restricted binding specificity, it directly binds to ErbB-4, but not to ErbB-1, ErbB-2 or ErbB-3. A similar selective binding to ErbB-4 has also been reported for NRG-3 (Zhang *et al.*, 1997) and may indicate that during development and in the adult, ligands with restricted ErbB specificities may play important roles. It is interesting to note that NRG-3 is the EGF-like ligand closest to NRG-4 (42% sequence identity in the EGF-like domain). Also relevant is the emerging wider repertoire of ErbB-4-specific ligands, as compared with growth factors that bind to ErbB-3. In addition to NRG-1, NRG-2, and NRG-3, ErbB-4 also binds to betacellulin (Riese *et al.*, 1996a), epiregulin (Shelly *et al.*, 1998) and HB-EGF (Elenius *et al.*, 1997). Moreover, at high ligand concentrations, or in the presence of a co-expressed ErbB-2, ErbB-4 also binds EGF and TGF α (Shelly *et al.*, 1998; Wang *et al.*, 1998). The broader specificity of ErbB-4 was reflected also in mutagenesis experiments: more NRG-1 mutants displayed greater affinity loss for ErbB-3 compared with ErbB-4 (Jones *et al.*, 1998).

Besides specificity to ErbB-4, NRG-3 and NRG-4 share relatively low affinity to this receptor compared with NRG-1 (Figure 4 and Zhang *et al.*, 1997). Several other ligands, such as epiregulin (Shelly *et al.*, 1998) and the alpha isoform of NRG-1 (Tzahar *et al.*, 1994), also display relatively low affinity to ErbB-4. These observations may suggest the existence of additional, yet undiscovered ErbB proteins, serving as high affinity receptors for these low affinity ligands. Alternatively, low affinity ligands may have a different biological function than high affinity growth factors, as they can escape the common rapid endocytic clearance from the extracellular space (Reddy *et al.*, 1996; Shelly *et al.*, 1998). Alternatively, the ligand-less co-receptor of ErbB-4, namely ErbB-2 (Karunakaran *et al.*, 1996), may be more effective in the case of low affinity ligands, such as NRG-3 and NRG-4, thus offering a mechanism for fine-tuning of ErbB signaling. The interaction of ErbB ligands with ErbB-2 appears to involve direct binding to an ErbB-2 promiscuous binding site (Klapper *et al.*, 1997; Tzahar *et al.*, 1997). According to this model, all EGF-like growth factors are bivalent ligands, that differ in their binding specificity to specific pairs of ErbB receptors (Tzahar *et al.*, 1997). This hypothesis may explain the multiplicity of ErbB ligands in terms of their differential ability to stabilize homo- and heterodimeric ErbB proteins. When applied to NRG-4, the bivalence model predicts that this ligand may differ from other ErbB-4-specific ligands, including NRG-3, in the ability to recruit heterodimer partners to ErbB-4.

Consistent with this model, we demonstrate that when co-expressed with ErbB-4, NRG-4 can recruit

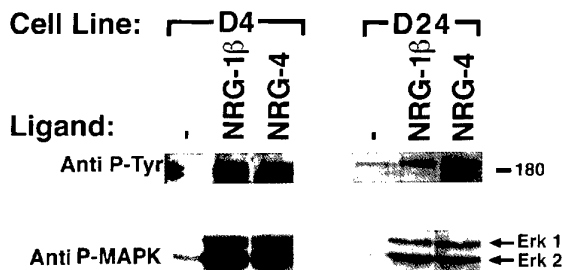


Figure 5 Tyrosine phosphorylation and MAPK activation by NRG-4. Derivatives of 32D cells expressing ErbB-4, either alone (D4 cells) or in combination with ErbB-2 (D24 cells) were incubated for 5 min at 37°C with either NRG-4 or NRG-1 β (each at 100 ng/ml). Whole cell extracts were then prepared, resolved by gel electrophoresis, and transferred to a nitrocellulose filter. The upper portion of the filter was immunoblotted with antibodies to phosphotyrosine (P-Tyr, the 150–200 kDa region is shown) or an antibody directed at the activated doubly phosphorylated form of the MAPK (Erk1 and Erk2, the 40–50 kDa region is shown). Antibodies were incubated with a secondary reagent coupled to horseradish peroxidase, allowing subsequent detection by chemiluminescence

both ErbB-1 and ErbB-2 into heterodimers (Figure 4b). These NRG-4-induced heterodimeric complexes may be of physiological importance, as indicated in proliferation assays: NRG-4 weakly stimulated the growth of myeloid cells engineered to express ErbB-4 alone (D4 cells). In contrast, this response was significantly enhanced upon ErbB-2 co-expression (D24 cells, Figure 3) when compared to that of the internal NRG-1 control. This finding may indicate that under some physiological conditions, the expression of ErbB-4 alone may be insufficient to elicit a biological response to NRG-4, requiring a co-receptor such as ErbB-2 to transduce its signal. This scenario has a precedence in the case of NRG-1: *in vitro* experiments showed clear enhancement of an ErbB-4-mediated mitogenic effect by a co-expressed ErbB-2 (Wang *et al.*, 1998), and gene-targeting in mice indicated that ErbB-2 is essential for cardiac trabeculation that is mediated by NRG-1 and ErbB-4 (Lee *et al.*, 1995).

With the exception of EGF, which is found in high concentrations in body fluids such as milk, urine and saliva (Carpenter and Cohen, 1979; Gregory *et al.*, 1979), all of the EGF/NGR family members are thought to act as short-range ligands affecting only neighboring cells through paracrine or autocrine loops (reviewed in Ben-Baruch *et al.*, 1998). Consistent with short-range ligand-receptor interactions, NRG-3 is expressed primarily in the central nervous system, along with its only known receptor, ErbB-4 (Plowman *et al.*, 1993; Zhang *et al.*, 1997). However, ErbB-4 is expressed also in muscle, heart, pancreas, salivary gland and lung (Gassmann *et al.*, 1995; Pinkas-Kramarski *et al.*, 1997; Plowman *et al.*, 1993). Our Northern blot analysis (Figure 2) demonstrated that in the adult, two of these ErbB-4-positive tissues, pancreas and muscle, express three molecular weight species of NRG-4. Likewise, multiple mRNA species of NRG-1 and NRG-2 were reported (Chan *et al.*, 1995; Wen *et al.*, 1992). Whether or not the multiplicity of NRG-4 mRNAs is related to the existence of many isoforms of NRG-1 and NRG-2 (Busfield *et al.*, 1997; Carraway *et al.*, 1997; Chang *et al.*, 1997; Marchionni *et al.*, 1993; Wen *et al.*, 1994) is currently unknown.

In summary we describe here the first characterization of NRG-4, a novel member of the ErbB ligand family, whose structure, expression pattern and restrained receptor-binding properties suggest a unique physiological role. Gene-targeting and *in vitro* studies with recombinant NRG-4 may resolve the presumed distinct biological role of this growth factor and its relationship to other EGF/NGR family ligands.

Materials and methods

Materials

EGF (human, recombinant) was purchased from Boehringer Mannheim. Recombinant human NDF β ₁₇₇₋₂₄₆ (NRG1- β 1) was obtained from Amgen (Thousand Oaks, CA, USA). Iodogen and bis(sulfosuccinimidyl) suberate (BS³) were from Pierce. Monoclonal antibodies (mAbs) to ErbB proteins (Chen *et al.*, 1996; Klapper *et al.*, 1997) were used for immunoprecipitation. The composition of buffered solutions was described (Tzahar *et al.*, 1994). Recombinant soluble extracellular domains of the four ErbB proteins (denoted IgB-1 through -4) (Chen *et al.*, 1996), in the form of fusion proteins containing the Fc portion of human immunoglobulin

G (IgG) were harvested from serum-free conditioned media of transfected HEK-293 human embryonic kidney cells. The PY20 antibody was purchased from Santa Cruz Biotechnology. A mAb to the active form of the MAP kinase (Yung *et al.*, 1997) was a gift from R Seger (Weizmann Institute).

Peptide synthesis

The EGF-like domain of NRG-4 (residues 4–50) was synthesized on an Applied Biosystems (ABI) 430A peptide synthesizer using standard *tert*-butoxycarbonyl (*t*-Boc) chemistry protocols as described (Barbacci *et al.*, 1995). Acetic anhydride capping was employed after each activated ester coupling. The peptide was assembled on phenylacetamidomethyl polystyrene resin using standard side chain protection, except for the use of *t*-Boc-Glu(O-cyclohexyl) and *t*-Boc-Asp(O-cyclohexyl). The peptide was deprotected using the 'Low-High' hydrofluoric acid (HF) method (Tam *et al.*, 1983). The crude HF product was purified by reverse phase HPLC (C-18 Vydac, 22 × 250 mm), diluted without drying into folding buffer (1 M urea, 100 mM Tris, pH 8.0, 1.5 mM oxidized glutathione, 0.75 mM reduced glutathione, 10 mM methionine), and stirred for 48 h at 4°C. The folded, fully oxidized peptide was purified from the folding mixture by reverse phase HPLC, and characterized by electrospray mass spectroscopy. A single HPLC peak with an averaged molecular mass (Mr) of 5371.50 was displayed by the reduced peptide prior to folding. This mass is in agreement with the theoretical Mr (5371.20). The folded and oxidized peptide displayed a slightly lower averaged molecular mass of 5366.88.

Database searches

EST databases were scanned for homology to the EGF-like domain of NRG-1 β (NDF- β) by Blast and Smith-Waterman algorithms (Samuel and Altschul, 1990; Smith and Waterman, 1981) using both a Unix-interfaced GCG server and a Bioaccelerator device (Compugen, Israel).

Northern blot

A Northern blot filter was purchased from Clontech (MTN Blot #7760-1), each lane containing approximately 2 μ g of poly(A)⁺ purified mRNA from healthy human tissues and run on a denaturing 1.2% formaldehyde/agarose gel. Hybridization to cDNA probes to mouse NRG-4 and human β -actin were performed with 'ExpressHyb' (Clontech) using the protocol provided by the manufacturer. Probing with a human amylase cDNA probe was performed by standard techniques. After each hybridization, blots were washed at room temperature for 40 min with several changes of low stringency wash solution (2 × SSC, 0.05% SDS) and then with at least two changes of high stringency buffer (0.1 × SSC, 0.1% SDS) at 50°C for 40 min.

Lysate preparation for Western blot analyses

For receptor activation studies, derivatives of the 32D cell line were resuspended in phosphate-buffered solution (PBS) and incubated at 22°C for 15 min before adding growth factors and incubating for 5 min at 37°C. Cells were then pelleted and lysed in ice cold solubilization buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Noidet-P-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1.5 mM EDTA, 1.5 mM MgCl₂, 2 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml aprotinin and 10 μ g/ml leupeptin) and left on ice for 15 min. The whole cell extract was then cleared by centrifugation (12 000 g for 10 min at 4°C), immediately boiled in reducing gel sample buffer, and resolved by 10% SDS-PAGE before being

transferred onto nitrocellulose. Filters were blocked in TBST buffer (0.02 M Tris-HCl [7.5], 0.15 M NaCl, 0.05% Tween-20) containing 1% milk for 40 min at 22°C, blotted with primary antibodies in TBST overnight at 4°C, followed by conjugation with a secondary antibody linked to horseradish peroxidase and subsequent detection by chemiluminescence (Amersham Corp.).

Radiolabeling of ligands, covalent crosslinking and ligand displacement analyses

Growth factors were labeled with Iodogen (Pierce) as described (Karunakaran et al., 1995). Chemical crosslinking to Chinese hamster ovary (CHO) cells engineered to express different ErbB combinations have been performed essentially as described (Tzahar et al., 1996). Briefly, radiolabeled ligands (at 100 ng/ml) were incubated for 2 h with cell monolayers at 4°C. The chemical crosslinking agent BS³ (1 mM) was then added and the cells were further incubated for 45 min at 22°C. Mouse antibodies were first coupled to rabbit anti-mouse IgG and to protein A-Sepharose beads, and then they were incubated with cell extracts for 2 h at 4°C. Immunoprecipitated complexes were then washed three times with ice-cold SBN buffer (1% NP-40; 150 mM NaCl; 10% Glycerol; 1 mM EGTA, in 50 mM Tris-HCl, pH 7.4; 1 ml per wash) prior to heating (5 min at 95°C) in gel sample buffer, resolution by gel electrophoresis, transfer to nitrocellulose and autoradiography. For crosslinking with IgBs, after co-incubation of IgB-containing conditioned media with radiolabeled ligands, complexes were immunoprecipitated directly with Sepharose-protein A beads. For ligand displacement analyses, cell monolayers were washed once with binding buffer, and then incubated for 2 h at 4°C with radiolabeled NRG-1 β (5 ng/ml) and various concentrations of unlabeled ligands, as indicated. Non-specific binding was determined in the presence of a 100-fold molar excess of

the unlabeled ligand. Cells were then washed, lysed in a solution containing 0.1 M NaOH and 0.1% SDS, and radioactivity determined by use of a gamma counter.

Cell proliferation assays

The establishment of a series of interleukin 3- (IL-3-) dependent 32D myeloid cells expressing all combinations of ErbB proteins has been described (Alimandi et al., 1997; Pinkas-Kramarski et al., 1996; Shelly et al., 1998). Cells were maintained in RPMI medium with 10% fetal bovine serum (FBS) and dilute IL3-containing conditioned medium. Prior to proliferation assays, cells were washed three times in RPMI/FBS and plated (5×10^5 cells/ml; 0.1 ml/well) into 96-well flat-bottomed plates with the indicated ligand concentrations or with IL-3 (1:1000 dilution of conditioned medium). Cell survival was determined 24 h later, or after the indicated time intervals, by MTT assay, as previously described (Mosman, 1983). MTT (0.05 mg/ml) was incubated with the analysed cells for 2 h at 37°C. Living cells can transform the tetrazolium ring into dark blue formazan crystals, that can be quantified by reading the optical density at 540–630 nm after lysis of the cells with acidic isopropanol.

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The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors

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ABSTRACT The *erbB-2/HER2* oncogene is overexpressed in a significant fraction of human carcinomas of the breast, ovary, and lung in a manner that correlates with poor prognosis. Although the encoded protein resembles several receptors for growth factors, no high affinity ligand of ErbB-2 has so far been fully characterized. However, several lines of evidence have raised the possibility that ErbB-2 can augment signal transduction initiated by binding of certain growth factors to their direct receptors. Here, we contrasted these two models of ErbB-2 function: First, examination of a large series of epidermal growth factor (EGF)-like ligands and neuregulins, including virus-encoded ligands as well as related motifs derived from the precursor of EGF, failed to detect interactions with ErbB-2 when this protein was singly expressed. Second, by using antibodies that block inter-ErbB interactions and cells devoid of surface ErbB-2, we learned that signaling by all ligands examined, except those derived from the precursor of EGF, was enhanced by the oncoprotein. These results imply that ErbB-2 evolved as a shared receptor subunit of all ErbB-specific growth factors. Thus, oncogenicity of ErbB-2 in human epithelia may not rely on the existence of a specific ligand but rather on its ability to act as a coreceptor for multiple stroma-derived growth factors.

Cellular growth and fate determination are controlled by a large variety of extracellular ligands and specific cell surface receptors. The largest family of such receptors is that of the growth factor receptors with intrinsic tyrosine kinase activity (1). Type-1 tyrosine kinase receptors, also known as ErbB/HER proteins, comprise one of the better-characterized subfamilies of growth factor receptors, of which the epidermal growth factor (EGF) receptor (ErbB-1) is the prototype (reviewed in ref. 2). The four ErbB members form homo- and heterodimeric complexes on binding of EGF-like or neuregulin (NRG) ligands, and, thereby, their kinase activity is stimulated and intracellular signals are generated. Constitutive stimulation of these pathways through autocrine or other mechanisms is associated with several types of human cancer (3). Most relevant is the frequent overexpression, often as a result of gene amplification, of ErbB-2/HER2 in breast, ovary, lung, and other types of epithelial cancers (reviewed in refs. 4 and 5). In some tissues, this overexpression was correlated with poorer prognosis and a more aggressive tumor phenotype (6).

Although ErbB-2 shares extensive structural homology with other ErbBs both along the catalytic intracellular domain and in the extracellular putative ligand binding region, many attempts to identify stimulatory ligands specific to ErbB-2 have so far failed. For example, detection of an activity that

enhances ErbB-2 phosphorylation led to molecular cloning of the Neu differentiation factor (NDF) and heregulin, two of a dozen isoforms of NRG1, all of which bind to ErbB-3 and ErbB-4 (7). Nevertheless, several observations imply that ErbB-2 homodimers, the plausible outcome of a direct ligand, may be functional *in vivo*. An oncogenic mutation that activates ErbB-2 phosphorylation apparently stabilizes such homodimers (8), and bivalent anti-ErbB-2 antibodies are mitogenic because they, like a direct ligand, dimerize ErbB-2 on the cell surface (9).

In parallel with attempts to isolate a direct ligand, several approaches culminated at the possibility that ErbB-2 functions, at least in part, as a coreceptor. Thus, coexpression of ErbB-2 together with ErbB-1 enhanced EGF-induced mitogenesis (10), and ErbB-2 presence reconstituted an extremely potent proliferative activity of ErbB-3, which is totally inactive when singly expressed (9). Consistent with its transactivating capability, ErbB-2 was found to act as the preferred partner of ligand-driven ErbB heterodimers (11, 12). The use of intracellular antibodies to ErbB-2 (13) has led to the conclusion that it can enhance signaling by two growth factors, EGF and NDF, through an ability to decelerate their release from the direct receptors, namely ErbB-1 and either ErbB-3 or ErbB-4, respectively (14).

Does ErbB-2 function as a high affinity receptor for a still unknown ligand of the EGF/NRG families, or could it act solely as a shared receptor subunit that amplifies signaling by prolonging the action of heterologous ligands? The present study addressed this question by using two strategies: First, we examined ligands that have not been previously tested for direct interaction with ErbB-2. On the other hand, we analyzed the generality of the transactivation ability of ErbB-2 by combining most existing ErbB ligands with mAbs that block a putative ligand binding site of ErbB-2. Our results strongly support the possibility that ErbB-2 evolved as a pan-EGF/NRG receptor rather than a high affinity receptor for a novel ligand. The implications of this scenario to epithelial tumors overexpressing ErbB-2 and to their inductive interactions with the underlying mesenchyme are discussed.

MATERIALS AND METHODS

Materials, Cell Lines, and Antibodies. The construction and sources of recombinant and synthetic growth factors were as previously specified (15, 16, 17). Recombinant soluble extra-

Abbreviations: EGF, epidermal growth factor; NDF, Neu differentiation factor; NRG, neuregulin; SFGE, Shope fibroma virus growth factor; proEGF, precursor of the epidermal growth factor; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; IgB, ErbB extracellular domains fused to an Fc portion of human Ig G. [‡]Present address: Procter and Gamble, Edificio Alvares Carbal, Quinta da Fonte, Porto Salvo 2780, Oeiras, Portugal.

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cellular domains of ErbB proteins fused to the Fc portion of human immunoglobulin G (IgB) have been described (18). Antibodies directed against ErbB-2, used for receptor activation and immunoprecipitation, have been described (19), as have those against ErbB-3 and ErbB-4 (18). An antiphosphotyrosine mAb (PY-20) was purchased from Santa Cruz Biotechnology. A mAb to the active form of the mitogen-activated protein kinase (MAPK) (20) was a gift from R. Seger (Weizmann Institute). T47D human breast cancer cells and their derivative T47D-5R have been described (13). 32D myeloid cells that ectopically express ErbB receptors have been described (9).

Expression of Recombinant Precursor of EGF (proEGF) Fusion Proteins. Four fragments containing the EGF-like domains of proEGF were constructed by PCR reactions on the full-length cDNA sequence of human proEGF in the pHEGF502 vector (kindly provided by Graeme I. Bell, Howard Hughes Medical Institute, Chicago) (21). The fragments, denoted pro1–4 (amino acids 314–479), pro5–8 (amino acids 741–952), pro5–9 (amino acids 741–1023), and EGF (amino acids 970–1023) were inserted into the pGEX expression vector (Amersham Pharmacia). Bacteria transformed with the constructs were induced to express the proteins and were harvested and lysed. Centrifugation-cleared lysates were mixed with glutathione-agarose beads and were incubated at 4°C while gently shaking. Elution of the bound proteins was carried out with 15 mM reduced glutathione and was followed by dialysis against PBS.

Cell Lysate Preparation. Cells grown as monolayers were solubilized as described (19). Proteins were separated electrophoretically either directly or after immunoprecipitation, were transferred to a nitrocellulose membrane, and were detected by immunoblotting.

Determination of Tyrosine Phosphorylation and MAPK Activation. Cells were incubated in PBS containing various ligands or mAbs at 37°C for the indicated time intervals. The treatment was ended by washing with ice-cold PBS. Whole cell lysates or immunoprecipitates were immunoblotted with an antiphosphotyrosine antibody (PY-20) or with a mAb that recognizes the doubly phosphorylated form of MAPK (20).

Cell Proliferation Assays. Proliferation of IL-3-dependent 32D cells expressing ErbB proteins was determined as described (9).

RESULTS

ErbB Ligands Cannot Activate a Singly Expressed ErbB-2, but Multiple Growth factors Can Activate it in Epithelial Cancer Cells. The ability of ErbB-2 to serve as a surrogate receptor when coexpressed with other family members, as well as the so-far unsuccessful search for a specific ErbB-2-binding ligand, suggest that its importance may reside in an intrinsic capacity to enhance signaling by a vast majority of ErbB-stimulating ligands. To experimentally test this scenario, we used an engineered 32D myeloid cell line that originally expresses no ErbB protein (9) and a large variety of known ErbB ligands (either EGF-like or NRGs). 32D cells that singly express ErbB-2 (D2) were incubated with growth factors, and the stimulation of ErbB-2 was followed by examining its phosphorylation on tyrosine residues (Fig. 1A). None of the 10 ligands tested was able to stimulate ErbB-2. That the protein is stimutable under these conditions was evident from the ability of a mAb to ErbB-2 [L140 (19)] to stimulate tyrosine autophosphorylation. Antibody bivalency is essential for kinase stimulation (19), indicating that homodimerization of ErbB-2, a bona fide attribute of a direct ErbB-2 ligand, is functional in D2 cells. By contrast with their inability to stimulate a singly expressed ErbB-2, all 10 ligands we examined stimulated ErbB-2 phosphorylation to different extents in SKOV-3 ovarian cancer cells (Fig. 1B), which express ErbB-2

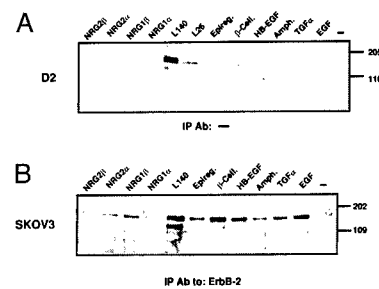


FIG. 1. ErbB-2 activation depends on coexpression of other ErbB proteins. ErbB-2 phosphorylation was determined in cells expressing the receptor singly (A, D2) or in combination with ErbB-1 and ErbB-3 (B, SKOV3). The indicated ligands (100 ng/ml) or antibodies (20 μ g/ml) were used to treat the cells for 5 min at 37°C. Receptor activation in whole cell lysates (A) or immunoprecipitates of ErbB-2 (B) was determined by an antibody directed against phosphorylated tyrosine.

along with ErbB-1 and ErbB-3. To exclude dependence on cell type, we also examined Chinese hamster ovary cells, which express ErbB-2 in the absence of other ErbB members, and T47D breast cancer cells that express all four ErbBs. Similar to the results presented in Fig. 1, none of the growth factors tested was able to activate ErbB-2 in the former, but all ligands were active on the latter cell type (data not shown). In conclusion, although homodimeric stimulation of ErbB-2 is achievable, its activation by hitherto identified ErbB ligands strictly depends on coexpression of other receptor partners.

ErbB-2 Augments Stimulation of Mitogenesis by Multiple ErbB Ligands. Because ErbB-2 can enhance signaling by NDF and EGF (14) and it is the preferred heterodimerizing partner of the respective receptors (11, 12), we hypothesized a similar role for this receptor in the transmission of signals by the majority of ErbB ligands. To examine the involvement of ErbB-2 in signaling by additional ligands, we applied mAbs that can inhibit ErbB-2 interactions with its family members [class II mAbs (19)] and 32D myeloid cells expressing defined ErbB combinations (9, 15). When deprived of IL-3, these cells totally depend on exogenous growth factors for survival. Cells expressing ErbB-2 with either ErbB-1 (D12), ErbB-3 (D23), or ErbB-4 (D24) were stimulated by EGF-like ligands in the presence of ErbB-specific mAbs. NRGs of several isoforms (NRG1 α , NRG1 β , and NRG2 α) induced cellular proliferation by promoting complexes containing ErbB-2 in combination with either ErbB-3 or ErbB-4 (Fig. 2; data not shown). This effect could be significantly decreased by anti-ErbB-2 antibodies capable of heterodimer destabilization (L26 and L96), as well as by their monovalent fragments (F26). mAbs directed against different epitopes (L87, L140, and L431) were incapable of exerting a similar effect, suggesting that interreceptor interactions, stimulated by all of the examined ligands, depend on a similar domain of ErbB-2. Inhibition of mitogenicity stimulated in cells coexpressing ErbB-2 with ErbB-3 was marked and similar in extent to that achieved by a ligand-displacing antibody directed against ErbB-3 [mAb C105 (18)]. Mitogenic stimulation by ligands that primarily stimulate ErbB-1 exhibited a similar pattern of ErbB-2 dependency (Fig. 2, lower panels). As previously demonstrated for EGF (19), the L26 antibody inhibited proliferation induced by transforming growth factor α in D12 cells. Both betacellulin and epiregulin, which benefit from ErbB-2 participation in their signaling (15, 22), induced a decreased mitogenicity in the presence of mAb L26 in D23 and in D24 cells, respectively. Taken together, the results shown in Fig. 2 indicate that ErbB-2 is capable of increasing ligand-stimulated mitogenicity without discriminating between the heterodimerizing ErbBs and their respective ligands.

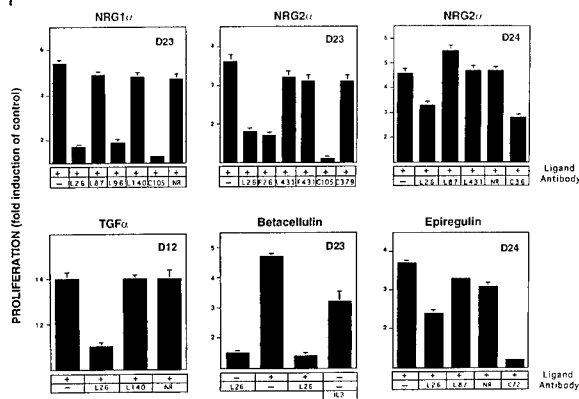


FIG. 2. ErbB-2-dependency of growth stimulation by EGF-like ligands. 32D cells expressing ErbB-2 with either ErbB-1 (D12), ErbB-3 (D23), or ErbB-4 (D24) were tested for cell proliferation. Cells deprived of IL-3 were treated with the indicated ligands. Anti-ErbB-2 mAbs belonging to class I (L431), class II (L26, L96), class III (L140), and class IV (L87) or their respective Fab fragments (F26, F431) were added simultaneously. Alternatively, control antibodies were used, including an unrelated mAb (NR), mAbs capable of ligand displacement from ErbB-3 (C105) or ErbB-4 (C72, C36), or an antibody against ErbB-3 that is incapable of displacing NRGs (C379). The extent of cell proliferation was determined 24 h after the addition of stimulating factors by using the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. The results are presented as fold induction over control untreated cells and are the mean \pm SD of eight determinations. Note that most mAbs (e.g., L26) have a weak agonist activity of their own.

ErbB-2 Enhances and Prolongs Signal Transduction by Multiple Growth Factors. Despite complexity of the ErbB signaling network, achieved by multiplicity of both ligands and receptors, signaling is funneled into a major cascade involving activation of the MAPK pathway. Recruitment of this pathway by an oncogenic ErbB-2 is essential for transformation (23), and ErbB-2 is known to augment signaling by EGF and NDF through MAPK (14). To pursue whether ErbB-2 involvement is a common cardinal element in signals promoted by ErbB ligands other than EGF and NDF, we used a breast cancer cell line, T47D, expressing all ErbB receptors and its derivative, T47D-5R, devoid of ErbB-2 surface expression due to intracellular entrapment (13). As demonstrated in Fig. 3, the parental cell line is induced, by different ligands, to activate the MAPK cascade, as determined by the detection of its two activated forms (20). Concomitant phosphorylation of a 180-kDa protein ensured the correlation between ErbB activation and subsequent events (shown for NRG1 and NRG2 α). Comparing the kinetics of activation to that in cells lacking surface ErbB-2 revealed a significant inhibition of intracellular activation in the latter. Both receptor phosphorylation and MAPK activation were affected. Stimulation by NRGs was decreased in duration as well as in intensity in cells lacking surface ErbB-2. Likewise, transforming growth factor α , although capable of inducing a similar increase in MAPK phosphorylation to that in the parental cells, showed a significant reduction in activation kinetics in T47D-5R cells. Stimulation by an additional ErbB-1-activating ligand, epiregulin, was affected in a similar manner to that of NRGs, decreasing to a barely detectable level in the absence of surface ErbB-2. To validate adequate expression of ErbB receptors in the 5R derivative, their amount was compared with that in the parental strain (data not shown): ErbB-1, ErbB-3, and ErbB-4 exhibited unaltered expression in T47D-5R cells. ErbB-2, in these cells, showed a characteristic faster electrophoretic migration, confirming its retention in the endoplasmic reticulum (13). In conclusion, expression of ErbB-2 at the cell surface can significantly prolong signaling by several growth factors, sug-

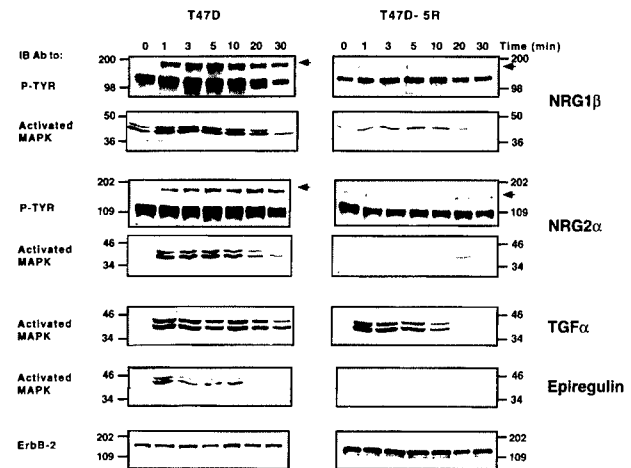


FIG. 3. The effect of surface-expressed ErbB-2 on the kinetics of ligand-induced tyrosine phosphorylation and MAPK activation. ErbB ligands were used to stimulate T47D breast cancer cells and their derivative, T47D-5R, which lacks surface expression of ErbB-2. A comparable number of cells was stimulated at 37°C by the indicated ligands (at 100 ng/ml) for various time intervals. Receptor activation, in whole cell lysates, was detected by immunoblotting (IB) with an antibody directed against phosphorylated tyrosine (P-TYR). MAPK activation in the same preparations was determined by using an antibody against the active doubly phosphorylated form of Erk proteins (Activated MAPK). For control of equal gel loading, the upper part of membranes used to detect MAPK was used to determine the amount of ErbB-2. Note that the 5R cells exhibited up-regulation of the cell-retained ErbB-2.

gesting a pan-ErbB stimulatory effect that is independent on ligand identity.

proEGF-Derived Units Are Unable to Recognize ErbB-2. Because the extracellular domain of ErbB-2 is homologous to the ligand-binding domains of other ErbB proteins and because all ErbB ligands share an EGF-like motif (24), an ErbB-2-specific ligand, if it exists, may include an EGF-like domain. Other possibilities, such as binding of a non-EGF-like ligand to a distinct site of ErbB-2, cannot, however, be excluded. The precursor of EGF, which shares transmembrane topology with most other precursors of ErbB ligands, includes nine EGF-like motifs, of which only the membrane proximal unit is an established growth factor (i.e., EGF). To examine whether other proEGF domains might harbor a capacity to recognize ErbB-2, we studied their functionality as separate fragments. Four recombinant fragments were designed: EGF-like domains I-IV (pro1-4), domains V-VIII (pro5-8), domains V-IX (pro5-9), and domain IX. The latter corresponds to the active unit, namely EGF, and served as a positive control. These protein fragments, as well as the analogous functional domain of NRG1 α (NDF) were expressed in bacteria in the form of glutathione *S*-transferase (GST) fusion proteins. To ensure correct expression and folding of the putative ligands, the functional domains of both EGF (GST-EGF) and NDF (GST-NDF) were tested for binding *in vitro* to soluble ErbB receptors [IgBs (18)]. Binding of the soluble receptors, denoted IgB1 through IgB4, to glutathione agarose-immobilized ligands confirmed that both GST-EGF and GST-NDF retained their receptor specificity (Fig. 4A Upper). Examining domains of proEGF in a similar manner could not reveal any novel recognition (Fig. 4A Lower), although the recombinant proteins exhibited the correct molecular weights and reacted with antibodies directed to respective peptides (data not shown). That failure to detect interaction *in vitro* was not caused by protein misfolding was implied by the retention of IgB1 binding by the pro5-9 recombinant protein consisting of the functional domain IX (Fig. 4A Lower). The absence of this domain, as in the case of pro1-4 and pro5-8 proteins,

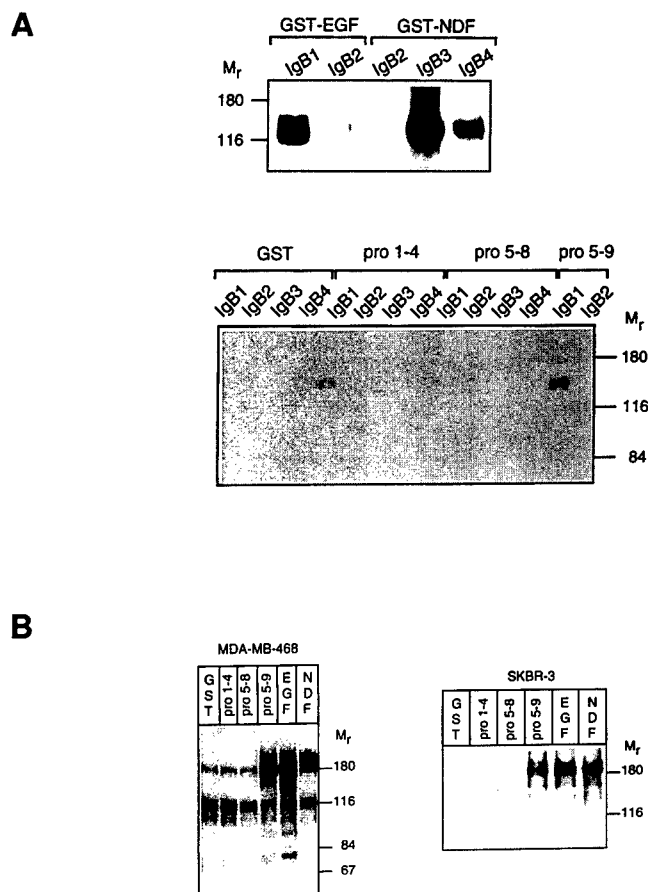


FIG. 4. Activation of ErbB receptors by EGF-like motifs of human proEGF. **(A)** GST fusion proteins containing EGF-like motifs 1–4, 5–8, or 5–9 of the EGF precursor were immobilized on glutathione-agarose beads. For control, GST fusion proteins containing EGF or NDF were used. The beads were incubated for 1 h at 4°C with conditioned media containing 1 μ g of the indicated IgB protein. Protein complexes were immunoblotted with an anti-human Fc antiserum for detection of bound IgBs. **(B)** Monolayers of the indicated human breast cancer cell lines were incubated, for 10 min at 37°C, in the presence of 100 ng/ml GST fusion proteins or 5 ng/ml ligands (EGF or NDF). Receptor activation was detected by an antiphosphotyrosine antibody.

abolished recognition, reinforcing its sufficiency for receptor binding. Moreover, none of the fragments could recognize any other ErbB protein, although IgB3 and IgB4 bound NRGs, and IgB2 bound all tested mAbs to ErbB-2 (Fig. 4A; data not shown).

The inability of proEGF-derived units to act as ErbB-binding ligands was evident also from experiments performed with living breast cancer cells (Fig. 4B). By detecting phosphorylation of proteins on tyrosine residues in whole cell lysates, we could demonstrate a pattern of receptor activation which is in accordance with the above binding. Only fragments containing domain IX could activate phosphorylation of proteins corresponding to ErbB receptors. Moreover, comparing an ErbB-2 overexpressing cell line (SKBR-3) with one devoid of the receptor (MDA-MB-468) revealed a similar specificity of stimulation, namely the dependence of activation on the ninth EGF-like domain. Collectively, these results indicate that no other EGF-like domain derived from the precursor molecule could serve as an ErbB-2-specific ligand.

ErbB-2 Is Activated by Three Viral Ligands only when Coexpressed with Other Family Members. Three EGF-like ligands encoded by poxviruses have been shown to resemble ErbB-activating molecules in structure as well as in activity. These ligands, including the vaccinia virus growth factor, the

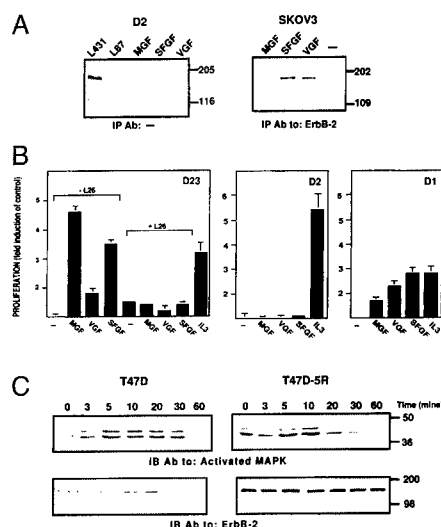


FIG. 5. Viral peptides recruit ErbB-2. **(A)** Phosphorylation of ErbB-2 by viral peptides [vaccinia virus growth factor (VGF), Myxoma virus growth factor (MGF), and SFGF] and antibodies (L87, L431) was examined as described in the legend to Fig. 1. **(B)** IL-3-deprived D23 cells were stimulated by viral peptides in the presence (+L26) or absence (–L26) of a class II mAb to the human ErbB-2 (Left). Cells singly expressing ErbB-2 (D2) or ErbB-1 (D1) served as negative and positive controls for ligand activity, respectively. Proliferation induction was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay as described in the legend to Fig. 2. For control of endogenous proliferation signals, we incubated cells with IL-3. **(C)** The effect of ErbB-2 on downstream activation by SFGF was examined in cells that do (T47D) or do not (T47D-5R) express ErbB-2 on their surface. A time response of activation was detected in whole cell lysates by immunoblotting with an antibody against activated MAPK. The amount of ErbB-2 was verified by immunoblotting the upper part of the membrane with an antibody against the receptor.

Shope fibroma virus growth factor (SFGF), and the Myxoma virus growth factor, harness the proliferation-inducing activity of ErbB receptors for the enhancement of their virulence (25). Synthetic analogs of these three viral ligands revealed specific patterns of ErbB specificity. For example, SFGF acts as a pan-ErbB ligand whereas Myxoma virus growth factor is more specific to the ErbB-2/ErbB-3 complex (17). Because evolutionarily the ErbB family evolved from a single protein whose ortholog in nematodes is Let-23 (26), and because it is likely that poxviruses coevolved with their vertebrate hosts (25), we assumed that an ErbB-2-specific ligand, if it ever existed, may have been retained in the genome of this large family of DNA viruses. To examine direct interaction between ErbB-2 and the three viral ligands, we used 32D cells singly expressing the protein (D2). As demonstrated in Fig. 5, none of the three known viral ligands promoted homodimerization of ErbB-2 and the consequent kinase activation (Fig. 5A Left) or mitogenic effect (Fig. 5B Center), although both activities were displayed by a mAb specific to ErbB-2 (Fig. 5A; data not shown).

Nevertheless, by using similar approaches to those presented above (Figs. 2 and 3), we learned that the three viral ligands, like their mammalian counterparts, depend on ErbB-2 for cellular activation. All three ligands could induce phosphorylation of ErbB-2 in SKOV3 cells (Fig. 5A Right), suggesting that the viral growth factors can recruit ErbB-2 into heterodimeric complexes. The involvement of ErbB-2 was also manifested biologically by a mitogenic assay (Fig. 5B); although none of the viral ligands was active on cells singly expressing the kinase-defective ErbB-3 receptor (data not shown), all three ligands potentially stimulated cells coexpressing it with the ligand-less ErbB-2 (Fig. 5B Left). Recruitment of

ErbB-2 by the viral ligands in these cells was evident also from the inhibitory activity of a class II mAb (L26) to ErbB-2 (Fig. 5B). Lastly, by using SFGF on T47D cells and the engineered 5R derivative, we observed an ErbB-2-mediated prolongation and enhancement of MAPK activation (Fig. 5C). Thus, although this ligand is capable of activating various ErbB complexes (17), it seems that SFGF, like the corresponding mammalian growth factors, depends on ErbB-2 as a coreceptor rather than as a direct high-affinity receptor.

DISCUSSION

Despite extensive investigation and a wealth of clinical data, the biochemical role of ErbB-2 in human cancer remains an enigma (4, 5). Although the structure and enzymatic function of the oncoprotein suggest that it is stimulated by a specific growth factor, *in vitro* studies along with the continuous failure to isolate a direct ligand imply a nonconventional receptor function (reviewed in ref. 27). This possibility has been strengthened by gene targeting experiments indicating cooperation between ErbB-2 and the neuregulin receptor ErbB-4 (28). By using a variety of ErbB ligands, our present study weakens the commonly held scenario arguing that ErbB-2 functions as an orphan receptor. Instead, a cooperative role in signal transduction is strongly supported.

The orphan receptor scenario predicts that an ErbB-2-specific ligand exists and that it contributes to tumor virulence by promoting homodimerization of the overexpressed ErbB-2 protein. However, contrary to this prediction, ErbB-2 homodimers that are driven either by a bivalent antibody (9) or by a point mutation (29) induce a mitogenic response that is weaker than that generated by ErbB-2-containing heterodimeric complexes. Another prediction made by the orphan receptor hypothesis is that the ErbB-2 ligand, if it exists, contains an EGF-like motif of six cysteine residues. However, it seems that no known EGF-like motif can directly bind to ErbB-2 with high affinity. For example, our most recent search for such an element in newly cloned EST databases identified one candidate, which we denoted NRG4 because the encoded protein exclusively binds ErbB-4 as its primary receptor (30). The EGF-like motif is found not only in ligand growth factors but also in cell adhesion proteins. For example, multiple copies of this domain are included in the extracellular matrix proteins laminin, tenascin, and thrombospondin, as well as in two *Drosophila* cell fate-determining proteins: Notch and Delta (reviewed in ref. 31). Our present results (Fig. 4), imply that all of the motifs included in proEGF, except the membrane proximal domain, belong to the second category of function. Indeed, modeling of the eight other motifs of proEGF, according to the published three-dimensional structure of EGF (32), indicated that domains 1–4 and 5–8 fall into distinct groups but that both groups significantly differ from the structures of EGF and NDF (M. Eisenstein, S.G., and Y.Y., unpublished results). Another important conclusion that emerged from the analysis of proEGF motifs is that the Gly-Xxx-Arg-Cys motif common to all ErbB ligands, but absent in nonligand motifs, may predict ErbB binding. Table 1 lists all of the currently known molecules that contain this motif, in the context of the EGF-like domain, along with their ErbB activating preference. Although it is clear that none binds to ErbB-2, it is also evident that signaling by all known ErbB ligands is enhanced by ErbB-2. This conclusion, along with the observation that certain anti-ErbB-2 antibodies can inhibit signaling by several NRGs and EGF-like ligands (Fig. 2), reinforces the possibility that ErbB-2 acts as a heterodimer partner rather than a direct receptor. Also supportive is the observation that each of the three other ErbB proteins serves as a direct receptor for more than one ligand (Table 1). It is therefore conceivable that, if ErbB-2 were able to bind a direct

Table 1. Receptor specificity of EGF-like ligands and neuregulins

| Ligand | 1 | 2 | 3 | 4 |
|---------------|-----|---|-----|-----|
| EGF | 1+2 | | 3+2 | 4+2 |
| TGF α | | | | |
| HB-EGF | | | | |
| Betacellulin | | | | |
| Amphiregulin | | | | |
| Epiregulin | | | | |
| NRG1 α | | | | |
| NRG1 β | | | | |
| NRG2 α | | | | |
| NRG2 β | | | | |
| NRG3 | | | | |
| NRG4 | | | | |
| VGF | | | | |
| SFGF | | | | |
| MGF | | | | |

All of the ErbB-stimulatory ligands are presented along with their ErbB preference. Interactions with the indicated ErbB homodimers (above diagonals) and the corresponding heterodimers with ErbB-2 (below diagonals) are indicated by using a color code: The most mitogenic interactions of each ligand are shown in black whereas white areas indicate absence of mitogenic signals. Note that ErbB-2 homodimers respond to no known ligand but that the mitogenic action of practically all growth factors can be augmented in the presence of ErbB-2. The data represent compilation of previous results obtained primarily with IL-3-dependent cells and the following ligands: NRG1s (9, 35), NRG2s (16), NRG3 (36), NRG4 (30), EGF (9, 22, 35, 37), transforming growth factor α (22, 37), epiregulin (15, 38), betacellulin (22, 39, 40), amphiregulin, and the viral ligands (17).

ligand, such a molecule would have been discovered, at least once.

In the absence of an ErbB-2-specific ligand, it may not be practical to test the prediction that ErbB-2 acts solely as a receptor subunit. However, the presence of genes encoding EGF-like ligands in the genome of poxviruses provided us an attractive opportunity to test this possibility. Like ErbB-2-overexpressing human carcinomas, the skin lesions induced by poxviruses display epithelial hyperproliferation and a transformed phenotype (25). Because poxviruses underwent coevolution with their mammalian hosts and were selected for efficient induction of epithelial lesions, it is reasonable to assume that an ErbB-2 ligand, if it existed, would have conferred a significant selective advantage to poxviruses that encoded it. Therefore, the observation that none of the three known viral growth factors can directly interact with ErbB-2 (Fig. 5) implies that this receptor may not be able to accommodate a specific ligand. On the other hand, ErbB-2 seems to fulfill a similar role in viral infection to that played in human carcinomas; the observed specificity of SFGF and especially Myxoma virus growth factor to the most mitogenic heterodimer, namely the ErbB-2/ErbB-3 combination (Table 1), suggests that poxviruses, much like carcinogenic mechanisms, gained the ability to harness the signal amplification ability of ErbB-2.

Perhaps the best exemplification of the capacity of ErbB-2 to transactivate signaling initiated by ligands binding to other ErbBs is the ability to reconstitute an extremely strong mitogenic activity of ErbB-3, a receptor whose homodimers are inactive (9). Because ErbB-3 is expressed by many carcinomas at moderately high levels and ErbB-2 is ubiquitously expressed, the cooperation between the two receptors is thought to drive or maintain the transformed phenotype of epithelial tumor cells (33). Examination of the molecular mechanism underlying

ing ligand-induced formation of this heterodimer may provide an explanation to the role played by ErbB-2 (34): Apparently, ErbB-2 can bind at very low affinity ligands like NRG1, but only when they are presented to it by their primary receptors. This model predicts that ErbB ligands are endowed with two binding sites and that the lower affinity site preferentially recognizes the putative binding cleft of ErbB-2, which may be the target of class II mAbs (19).

In conclusion, ErbB-2 emerges as a master coordinator of a signaling network rather than as a receptor that mediates the action of one specific ligand. The relative topology of ErbB proteins, which are situated primarily on the basolateral face of epithelial cells, and their respective ligands, which are synthesized by the underlying stromal cells, implies that ErbB-2 can act as an amplifier of signaling by all of the stromal ligands listed in Table 1. Complete sequencing of the human genome and characterization of the remaining EGF motif-containing genes will ultimately answer the question whether this is the only function of ErbB-2 or whether a still-unknown ligand that binds to it with high affinity does exist.

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Biochemical and Clinical Implications of the ErbB/HER Signaling Network of Growth Factor Receptors

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Carcinoma, cancer of epithelial cells, is a major cause of morbidity and mortality in Western societies. Clonal fixation and propagation of oncogenic genetic changes, sporadically accumulating in epithelial cells, depend on growth factors and their surface receptors. One of the large families of receptors is that of the ErbB tyrosine kinases, which bind multiple neuregulins and other epidermal growth factor-like molecules. Certain ErbB members and their ligands are involved in human cancers of various origins. However, most of the clinical data relate to ErbB-2, a protein whose overexpression in subsets of carcinomas can predict poor prognosis. Although no ligand has so far been assigned to ErbB-2, recent biochemical evidence implies that this oncoprotein operates as a shared receptor subunit of other ErbBs. Several biochemical attributes enable ErbB-2 to act as an epithelial cell amplifier of stroma-derived growth factor signals: It delays ligand dissociation, enhances coupling to the mitogen-activated protein kinase pathway, and impedes the rate of receptor downregulation. The realization that ErbB-2 is a master regulator of a signaling network that drives epithelial cell proliferation identifies this protein as a target for cancer therapy. Indeed, various ErbB-2-directed therapeutic approaches, including immunological and genetic therapies, demonstrate promising clinical potential. © 2000 Academic Press.

I. INTRODUCTION

Cellular transformation, underlying the promotion and progression of human tumors, is, to date, acknowledged as the result of cumulative independent mutations (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996; Nowell, 1976). Numerous players of a cellular network designed to convey proliferative signals are plausible and often established candidates for such transformation-driving mutations (Bishop, 1991). Among these, polypeptide growth factors, as well as their receptors, comprise a group of major activators of such cellular signaling pathways, enabling the influx of information and mediating its frequency and intensity, making these molecules prime suspects in the promotion of pathophysiology.

Activation of a growth factor receptor was first linked to human cancers via identification of the homology between the epidermal growth factor receptor (EGFR) and the viral oncogene *v-erbB*, encoded by an avian erythroblastosis retrovirus (Downward *et al.*, 1984). Cancers of the rat nervous system, induced by a chemical carcinogen, led to the discovery of an EGFR-related 185-kDa phosphoprotein, designated the Neu oncoprotein (Padhy *et al.*, 1982; Schechter *et al.*, 1984). A single point mutation, replacing a valine with a glutamic acid in the transmembranal region of the Neu oncoprotein, provided its transforming capability (Bargmann *et al.*, 1986). Independently, sequence similarity to the *erbB-1* gene, encoding for the EGFR, resulted in the isolation of the human ortholog of *neu*, HER2 (for human EGF receptor 2) or *erbB-2* (Coussens *et al.*, 1985; King *et al.*, 1985; Yamamoto *et al.*, 1986). Screening of genomic DNA and messenger RNAs with probes de-

rived from ErbBs allowed isolation of two additional relatives of the human *erbB-1* gene. These were named *erbB-3* (or HER3) and *erbB-4* (or HER4) (Kraus *et al.*, 1989; Plowman *et al.*, 1990, 1993a,b).

Early on, the similarity between ErbB-2 and ErbB-1 (the four ErbB proteins are compared in Fig. 1) suggested its activation by a direct ligand, and led to several attempts to isolate an ErbB-2 binding protein (Lupu *et al.*, 1990; Yarden and Peles, 1991; Yarden and Weinberg, 1989). These ventures yielded an ErbB-2 activating molecule termed neu differentiation factor (NDF) (Peles *et al.*, 1992; Wen *et al.*, 1992) or heregulin (Holmes *et al.*, 1992) only to be revealed later as the direct binding ligand of the two closely ho-

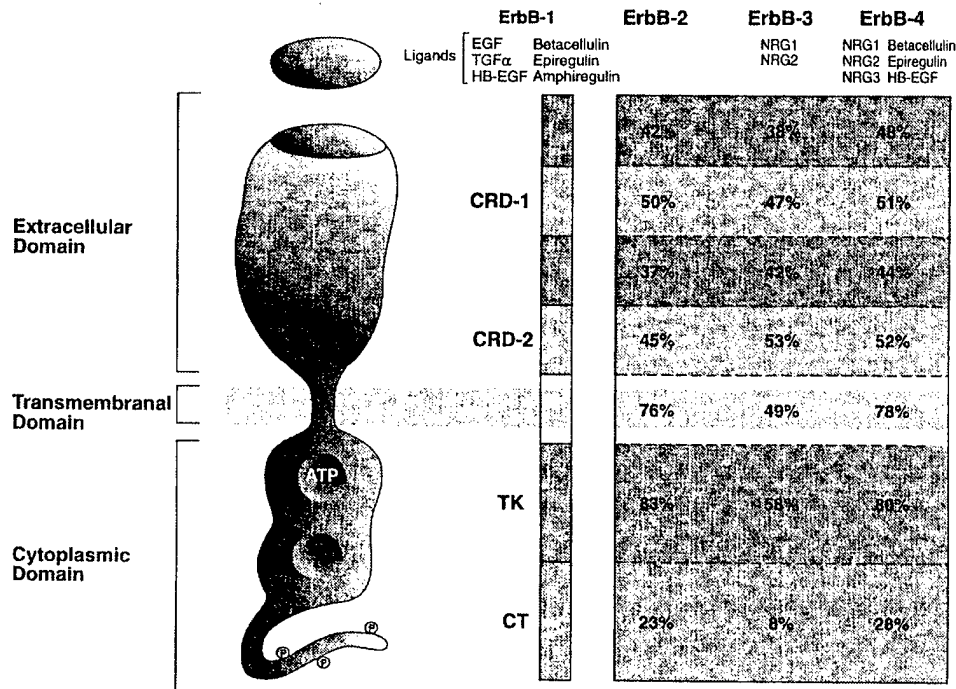


Fig. 1 The ErbB family of receptors. The prototypic protein for the four members of the family is represented as a bilobular membrane-spanning structure. An extracellular portion, stabilized by two cysteine-rich domains (CRD-1 and CRD-2), possesses the ligand binding capacity of the receptor. Its four zones show a high diversity between the family members as presented in percentage of homology to the first identified receptor, ErbB-1. This diversity confers specific primary recognition of multiple ligands as listed for each of the receptors. Specificity is extended by cross-reactivity of several of the ligands; NRG1 and NRG2 are shared by ErbB-3 and ErbB-4; betacellulin, epiregulin, and HB-EGF are recognized by both ErbB-1 and ErbB-4. ErbB-2 is ligandless. High homology of the cytoplasmic tyrosine kinase domain is altered in the case of ErbB-3 that is catalytically impaired. The C-terminal tail (CT), harboring docking sites for effector proteins of signaling pathways, exhibits a low similarity, implying a high diversity of interacting molecules.

mologous receptors, ErbB-3 (Carraway *et al.*, 1994; Kita *et al.*, 1994; Sliwkowski *et al.*, 1994; Tzahar *et al.*, 1994) and ErbB-4 (Plowman *et al.*, 1993a; Tzahar *et al.*, 1994), thus rendering ErbB-2 orphan of a known high affinity binding molecule. This contrasts with its family members capable of binding multiple ligands with some overlapping specificities (Fig. 1). Together, the four ErbB proteins comprise the first subtype of the family of receptor tyrosine kinases (RTKs) (van der Geer *et al.*, 1994). Typical to proteins of RTK affiliation, ErbB receptors bear an extracellular ligand binding domain, characterized by a relatively high diversity between family members and capable of transmitting a signal that results in the activation of the intracellular portion of the protein. Central to this activation is an intrinsic tyrosine kinase catalytic core showing close resemblance between the receptors, but wide variety in the flanking sequences enabling diversity of interactions with receptor-specific effector proteins. The latter are adapter molecules, some endowed with an enzymatic activity, that share one of several phosphotyrosine binding domains (e.g., a Src homology 2 domain). Receptor activation results in phosphorylation of specific tyrosine residues located within the receptor's cytoplasmic region, which leads to the recruitment of phosphotyrosine-binding effector proteins, and subsequent simultaneous stimulation of multiple signaling pathways. As with other allosteric enzymes, the monomeric RTK molecule is inactive but a dimeric form is fully active. The EGF-like ligands act as allosteric modifiers by promoting rapid receptor dimerization (Yarden and Schlessinger, 1987).

Despite their overall structural similarity, the four ErbBs display differences in structures of autophosphorylation docking sites, in substrate specificity, and in potency of the kinase activity. Most remarkable is the defective kinase function of ErbB-3 and the absence of a known ligand for ErbB-2, although the transforming ability of this receptor is higher than that of other ErbBs. The prerequisite of receptor oligomerization for the activation of ErbB kinases paved the way for the understanding of the capability of ErbB-2 to transform cells regardless of its orphanhood. It suggests that receptor dimerization, between identical or sibling molecules, can induce the activation of ErbB-2, independent of a specifically binding ligand. This network of receptor interactions serving to increase the diversity and robustness of signaling induced by the activation of ErbB receptors (Alroy and Yarden, 1997) operates as a major proliferation engine of several types of cells, of which most critical for cancer are the epithelial cells. This function places the additional three members of the family, alongside ErbB-2, in the center of cancer research interests. As expected from this model, concomitant expression of these receptors or their ligands is a common feature of many adenocarcinomas (Gullick, 1990; Hynes and Stern, 1994; Salomon *et al.*, 1995a; Stancovski *et al.*, 1994). It is, however, most dominantly characterized by a high incidence of the ErbB-2 protein, suggesting further that it is this protein that confers a pro-

liferative advantage to cells (Tzahar and Yarden, 1998). The mode by which ErbB-2 exerts this effect, driving cells toward a more transformed phenotype, is still an open question and will reside in the center of our discussion.

Commencing with the clinical aspects of ErbB proteins and their ligands, we proceed to discuss the mechanisms underlying their possible role in transformation. Specifically, the clinical significance of ErbB-2 is discussed in the context of a model that attributes receptor interactions to bivalent ligand binding, enabling receptor cooperativity. Moreover, the high transforming potential of ErbB-2 in epithelial tumors is considered in view of its ability to act as a shared low affinity receptor for the many stroma-derived EGF-like growth factors. Last, we debate the therapeutic opportunities developing through our better understanding of the role of ErbB receptors and their ligands in human cancer.

II. CLINICAL ASPECTS OF ErbB RECEPTORS

Under normal conditions the ErbB signaling module acts as a network that mediates the interactions between different cell types, such as the crosstalk between neurons and muscle fibers at the neuromuscular synapse, Schwann cell–neuron interactions in the peripheral nervous system, and endocardium to myocardium crosstalk in the developing heart (Burden and Yarden, 1997). These interactions are unleashed in tissues undergoing malignant transformation. The best example is the mesenchyme–epithelial interaction, which allows ErbB-expressing epithelial sheets to receive morphogenic cues from the stroma in the form of EGF- and neuregulin-like growth factor ligands (Threadgill *et al.*, 1995; Yang *et al.*, 1995). Several animal models of cancer display activated forms of the ErbB signaling network, such as a truncated ErbB-1, which is encoded by the viral *erbB* oncogene of the avian erythroblastosis virus (Downward *et al.*, 1984), a carcinogen-induced mutant of ErbB-2 that can promote Schwannomas in rodents (Bargmann *et al.*, 1986), and transcriptionally active autocrine loops involving TGF- α in several retrovirally transformed cells (Salomon *et al.*, 1995a). Apparently, such autocrine loops are operational in some types of human tumors, as described later, and aberrant forms of ErbB-1 are found in human neuroblastomas (see later discussion), but most relevant to human cancer is overexpression, often as a result of gene amplification, of ErbB-2. In normal epithelial cells, ErbB-2 is expressed at low levels, especially in embryonic stages (Kokai *et al.*, 1987), but a variable fraction of several types of epithelial cancers exhibits remarkably increased expression, up to 100-fold. The clinical implications of ErbB-2 overexpression are well described in breast and ovarian cancers, two tumor types that are the major focus of our discussion, but oth-

er types of tumors also exhibit high ErbB-2 expression, including tumors of the lung, salivary gland, kidney, and bladder. Here we discuss the most recent data related to ErbB-1, ErbB-3 and ErbB-4, and then concentrate on ErbB-2. The reader is referred to earlier reviews that cover specific aspects of the field (Salomon *et al.*, 1995a; Hynes and Stern, 1994; Gullick, 1990; Stancovski *et al.*, 1994).

ErbB-1 ErbB-1 expression is found more frequently in squamous cell carcinomas of the lung as opposed to other lung cancer histologies (Veale *et al.*, 1987), and it correlates significantly with high metastatic rate, poor differentiation, short patient survival time (Pavelic *et al.*, 1993), and poor prognosis (Volm *et al.*, 1992). Neoplasms such as renal, ovarian, and breast carcinoma display elevated expression of ErbB-1, but correlation with clinical outcome is inconclusive. For example, approximately 37% of invasive ductal carcinomas of the breast express ErbB-1, but correlation with prognosis is unclear: In a study of 309 breast cancer patients, no relation of ErbB-1 to disease-free or overall survival was noted (Charpin *et al.*, 1993). However, in node negative disease, ErbB-1 expression was a significant negative prognostic factor for relapse and survival (Harris *et al.*, 1992; Nicholson *et al.*, 1991). In addition, ErbB-1 expression significantly predicts for relapse in operable breast cancer patients (Gasparini *et al.*, 1994a), and is associated with shorter disease-free and overall survival in advanced breast cancer (Archer *et al.*, 1995). In a study assessing fine-needle aspirations on women at high risk for breast cancer, ErbB-1 was significantly more highly expressed in high-risk than in low-risk women, suggesting that it may be a useful marker in this subset of women (Fabian *et al.*, 1993, 1996). In bladder cancer, ErbB-1 expression correlates with stage and poor prognosis in patients with a transitional cell type (Berger *et al.*, 1987; Chow *et al.*, 1997; Korkolopoulou *et al.*, 1997; Lonn *et al.*, 1993; Nguyen *et al.*, 1994). It is also overexpressed in head and neck tumors (Irish and Bernstein, 1993), which are also squamous cell cancers, and tend to be prevalent within the same patient population as lung cancer. ErbB-1 mRNA overexpression, but not gene amplification, was noted in about 40% of benign prostatic hypertrophy cases (Schwartz *et al.*, 1998), while in prostatic carcinoma, overexpression of ErbB-1 was associated with poorer prognosis (Visakorpi *et al.*, 1992). In renal cell carcinoma, coexpression of ErbB-1 and ErbB-2 was significantly correlated with metastatic disease (Stumm *et al.*, 1996). Taken together, only non-small cell lung cancer (NSCLC) is presently considered a disease in which ErbB-1 can serve as a prognostic marker, but other types of carcinomas are likely candidates. The situation is different in brain malignancies because several types of clinically relevant alterations of the *erbB-1* gene were reported. Both EGF and its receptor are overexpressed in glial tumors (Libermann *et al.*, 1985). In fact, overexpression, often due to amplification of the *erbB-1* gene, is the most common genetic alteration in glial tumors, and it

correlates with poor prognosis (Wong *et al.*, 1992). Overexpression is more frequent in higher grade neoplasms and correlates with higher proliferation and reduced survival (Jaros *et al.*, 1992). Further, in a significant fraction of tumors showing overexpression the *erbB-1* gene displays rearrangements, resulting in in-frame deletions of portions of the extracellular domain of ErbB-1. The most common mutation (type III) deletes amino acids 6–273, yielding a protein whose ligand binding is defective, but it is constitutively phosphorylated and its tumorigenicity *in vivo* is enhanced (Nishikawa *et al.*, 1994).

ErbB-3 The third member of the ErbB family, ErbB-3, differs from the others in that it contains a nonfunctional tyrosine kinase domain, and thus it signals only as a heterodimer partner. ErbB-3 is normally expressed in many tissues other than the hematopoietic system, in a pattern differing from that of ErbB-1 or ErbB-2 (Prigent *et al.*, 1992). This pattern in normal tissue is paralleled in neoplastic processes as well. In breast cancer, several studies have shown that more than 50% of tumors will have some ErbB-3 positivity by immunohistochemistry, and about 20–30% will show strong membrane staining (Gasparini *et al.*, 1994b; Lemoine *et al.*, 1992a; Quinn *et al.*, 1994; Travis *et al.*, 1996). As opposed to the case of ErbB-2, no significant correlation between ErbB-3 overexpression in breast cancer and patient survival, tumor size, and recurrence was found. In gastrointestinal cancers, extensive overexpression of ErbB-3 seems to occur. For example, in gastric cancer, ErbB-3 was found to be more highly expressed than ErbB-2 and it was detectable in most tumors (Sanidas *et al.*, 1993). Similarly, a large number of pancreatic tumors express ErbB-3 (Lemoine *et al.*, 1992b) and 55% of colon tumors, as opposed to 22% of normal colon tissue, express the *erbB-3* mRNA (Ciardiello *et al.*, 1991). In oral squamous cell cancers, ErbB-3 overexpression was linked to lymph node involvement, invasion, and patient survival (Shintani *et al.*, 1995). ErbB-3 is overexpressed in prostate cancer, with high levels of membranous staining, which persists in metastatic disease (Myers *et al.*, 1994). Another difference between ErbB-2 and ErbB-3 as prognostic parameters is seen in ovarian cancer, where 85% of tumors stain positively, but with stronger staining seen in borderline and early stage tumors (Simpson *et al.*, 1995). In melanoma as well, ErbB-3 overexpression was found more frequently in nevi than in malignant states and was not seen in cases of metastatic disease (Korabiowska *et al.*, 1996). Taken together, these initial clinical correlates of ErbB-3 attribute to it a role in carcinoma development, which may differ from that of ErbB-2. Given the necessity of ErbB-3 heterodimerization, the true role of ErbB-3 overexpression as a prognostic factor might be better clarified if coexpression with ErbB-2 were analyzed. Indeed, in one study of papillary thyroid cancer 64% of tumors showed coexpression of ErbB-2, -3, and -4 (Haugen *et al.*, 1996).

ErbB-4 Only a few clinical studies of ErbB-4 are available. Consistent

with the abundant expression of this neuregulin receptor in the nervous system, a large fraction of pediatric neuroblastomas expresses ErbB-4 (Gilbertson *et al.*, 1997). Even more important, coexpression with ErbB-2 was found in 54% of tumors, but neuregulin expression was limited to 31% of cancers, and no correlation with clinicopathological disease features was observed. By contrast, in a survey of 24 prostatectomy specimens no tumor expressed neuregulin, but 100% of the stroma contained the ligand, and 23% of prostate cancer specimens expressed ErbB-4 (Lyne *et al.*, 1997). More studies on ErbB-4 involvement in cancer, and in particular its cooperation with ErbB-2, are indicated.

A. Breast Cancer

Unlike ductal carcinoma *in situ* (DCIS) and its probable outcome, infiltrating ductal cancers (IDC), which show relatively high amounts of the protein, ErbB-2 overexpression has not been found in benign breast disease (Allred *et al.*, 1992a; Gusterson *et al.*, 1988; Regidor *et al.*, 1995; Schimmelpenninck *et al.*, 1992). However, metastatic lesions arising from ErbB-2-overexpressing tumors maintain overexpression (Iglehart *et al.*, 1995; Niehans *et al.*, 1993), suggesting that ErbB-2 is not involved in premalignant stages, but its function may be essential for progression and metastasis.

1. DCIS

Ductal carcinoma *in situ* is defined as a ductal proliferation of malignant cells that have not invaded the basement membrane. Much attention has been given recently to attempting to improve classification and stratification of DCIS, because a great deal of uncertainty remains and a wide range of approaches is available for treatment and prognosis of this condition. The incidence has risen dramatically, from 5,000 cases in 1983 to more than 23,000 in 1992, according to the NCI SEER study (Ernster *et al.*, 1996), and it represents about 12% of all breast cancer (Kerlikowske *et al.*, 1997). Despite the recognition that at least 30% of these cases may progress to advanced disease, there is continued debate about which cases require aggressive intervention. Early on, it was recognized that the comedo form of DCIS often shows a greater degree of membranous staining for ErbB-2 than does the non-comedo form (Allred *et al.*, 1992a; Lodato *et al.*, 1990). In fact, it is estimated that up to 90% of comedo DCIS overexpress ErbB-2 (Barnes *et al.*, 1992; van de Vijver *et al.*, 1988). To better stratify risk among DCIS cases, different grading systems were developed. Moreno, breaking down DCIS into high grade versus low grade or intermediate grade, showed a significant difference in ErbB-2 expression between the high-grade DCIS and all others,

but not between low and intermediate grade (Moreno *et al.*, 1997). In a study of 127 cases of DCIS, 57% were found to be ErbB-2 positive, with overexpression found more frequently in less differentiated DCIS according to the grading system of Holland (Zafrani *et al.*, 1994). Mack *et al.* (1997) analyzed ErbB-2 levels in DCIS cases stratified according to the new histological classifications as described by Scott (Scott *et al.*, 1997) and found that the differences in ErbB-2 staining were significant when related to subclass (Mack *et al.*, 1997). It was found that even in analysis of invasive disease, the level of ErbB-2 expression depended on whether the *in situ* component was comedo or non-comedo (Brower *et al.*, 1995). Along the same line, 21% of axillary metastases were shown to have a revertant phenotype, to a DCIS identical to the original presentation, and in these cases ErbB-2 levels were identical to those predevelopment of IDC (Barsky *et al.*, 1997). Paget's disease, which is essentially an aggressive form of DCIS, overexpresses ErbB-2 (Bose *et al.*, 1996; Lodato *et al.*, 1990), while lobular carcinoma *in situ* does not (Fisher *et al.*, 1996; Lodato *et al.*, 1990; Porter *et al.*, 1991; Ramachandra *et al.*, 1990).

2. MALE BREAST CANCER

In two studies, one of 30 male breast cancer specimens and the other of 41, overexpression of ErbB-2 was similar to that detected in females (Bruce *et al.*, 1996; Willsher *et al.*, 1997). This differs from earlier results on 21 patients published by Fox *et al.* (1991) where no male breast cancer specimens were positive. These variant results highlight the need for standardization of techniques and reagents in order to document definitively the role of molecular markers in the clinical setting, because the current impression is that although male breast cancer is much less common, there are few molecular differences between male and female breast cancer (for example, male breast cancer is frequently estrogen and progesterone receptor positive), even if the etiology in the male is still enigmatic (Memon and Donohue, 1997; Wagner *et al.*, 1995).

3. CARCINOMAS

Studies show that overall about 30% of invasive ductal carcinomas manifest amplification of ErbB-2 (Lipponen *et al.*, 1993; Slamon *et al.*, 1987). No difference was seen in ErbB-2 expression between different ethnic groups in the United States (Elledge *et al.*, 1994; Weiss *et al.*, 1995). While DCIS of the comedo type more frequently shows higher expression than does invasive disease, tumors greater than 1 cm in size tend to show ErbB-2 overexpression more frequently than do smaller tumors (Schimmelpenninck *et al.*, 1992), and higher grade tumors more frequently overexpress than do lower

grade ones (Tervahauta *et al.*, 1991). Lobular carcinoma, similar to lobular carcinoma *in situ*, does not overexpress ErbB-2 (Gusterson *et al.*, 1992). The significance of the lower incidence of ErbB-2 overexpression in IDC compared with DCIS remains unknown. While some evidence supports the notion that comedo DCIS more often than other types progresses to IDC, the probable precursor-product relationships are still unclear. Nevertheless, it appears likely that invasion through the basement membrane selects malignant cells that do not overexpress ErbB-2, but later on those ErbB-2-overexpressing tumors benefit a proliferative advantage. Indeed, some recent observations in animal model systems and in breast cancer patients suggest that once a tumor is established in a distant organ, it becomes more engaged in proliferation and even reverts to its premetastatic phenotype (Barsky *et al.*, 1997).

4. MOLECULAR MARKERS, LYMPH NODE STATUS, AND OVERALL PATIENT SURVIVAL

ErbB-2 expression is related to the absence of the two steroid hormone receptors, ER and PR, in breast cancers (Gusterson *et al.*, 1992; Tandon *et al.*, 1989). Multiple studies have demonstrated a positive correlation between p53 expression and ErbB-2 expression in tumors ranging from DCIS to invasive ductal carcinoma (Lipponen *et al.*, 1993; Naidu *et al.*, 1998). In estrogen receptor positive tumors, the combination of p53 and ErbB-2 positivity predicts more undifferentiated carcinoma, the presence of axillary nodes, and shorter disease-free survival (Bebenek *et al.*, 1998; Wiltschke *et al.*, 1994). E-cadherin, the reduction of which is associated with increased invasiveness, was inversely related to ErbB-2 expression (Charpin *et al.*, 1997). The status of another marker of tumor invasion and metastasis, the acidic lysosomal proteinase cathepsin D, is less clear. Whereas one study found no relation between ErbB-2 expression and tumor cell levels of cathepsin D (Scorilas *et al.*, 1993; Tetu *et al.*, 1993), a correlation was noted in node positive patients (Seshadri *et al.*, 1994). Intriguingly, high levels of ErbB-2 in breast tumors correlate with increased cathepsin D in the surrounding stromal cells, which was predictive of shorter metastasis-free survival in chemotherapy-treated patients. Lastly, chromosome 1 aneusomy, as detected by FISH analysis, was significantly related to increased ErbB-2 expression (Farabegoli *et al.*, 1996).

One of the problems in clinical oncology is the determination of nodal metastases in breast cancer patients; if there were a marker that could predict this accurately, then more conservative surgery could be performed and more aggressive therapy reserved for those patients with more extensive disease. The identification of increased levels of ErbB-2 mRNA in fine-needle biopsy specimens was predictive of lymph node involvement preoperatively (Anan *et al.*, 1998). Several studies have shown positive correlation between

ErbB-2 overexpression and nodal metastases (Eissa *et al.*, 1997; Midulla *et al.*, 1995; Noguchi *et al.*, 1993; Tiwari *et al.*, 1992), but this was not confirmed by other studies (Rilke *et al.*, 1991). Although lymph node status per se is the major prognostic factor indicating future relapse, even in lymph node negative breast cancer 30% of patients will relapse. Thus, it is imperative to determine other prognostic indicators that could identify which patients may have a more aggressive disease that requires more intensive treatment. The amount of ErbB-2, especially when analyzed in relatively large groups of patients (>300 specimens), appears to predict clinical outcome in both node negative and node positive patients, and thus may serve as a useful indicator. For example, in node negative breast cancer, overexpression of ErbB-2 or p53 was predictive of a decreased overall survival as well as disease-free survival (Albanell *et al.*, 1996; Han *et al.*, 1997; O'Malley *et al.*, 1996; Paterson *et al.*, 1991; Sauer *et al.*, 1992). In some studies, the decreased overall survival was seen particularly in node positive patients (Rilke *et al.*, 1991; Slamon *et al.*, 1987; Tandon *et al.*, 1989; Toikkanen *et al.*, 1992; Tsuda *et al.*, 1998). Bertheau *et al.* (1998) showed that the status of ErbB-2 was prognostic by the Cox model analysis in the younger patient cohort, under age 35, whereas p53 was prognostic in the age 36–50 group. Others have shown that prognostic information was more reliable in the postmenopausal patient (Tervahauta *et al.*, 1991). In early stage breast cancer (stages I and early stage II) use of p53 and ErbB-2 in an artificial neural network analysis gave better predictive information than the TNM staging system, and was useful in predicting response to adjuvant chemo- and radiotherapy (Burke *et al.*, 1998). Some studies show that ErbB-2 overexpression is an independent prognostic factor for high-grade disease (Lipponen *et al.*, 1993), patient survival (in either univariate or multivariate analysis) (Eissa *et al.*, 1997; Lonn *et al.*, 1994, 1995; Noguchi *et al.*, 1993), regardless of nodal status (Gullick *et al.*, 1991). Other studies did not confirm these correlations (Clark and McGuire, 1991; Kury *et al.*, 1990). These and other conflicting results may relate to the size of the population analyzed and to some technical considerations. Essentially, determination of positivity may vary, especially in earlier studies, with the type of analysis or the identity of antibody used for ErbB-2 immunostaining. In addition, because ErbB-2 function may not be autonomous (see later discussion), the extent of coexpression of neuregulins and EGF-like stromal growth factors, as well as the expression of other ErbB proteins (especially ErbB-3), the status of ErbB-2 phosphorylation, and its membrane localization are all factors that may be critical for determining the significance of an overexpressed ErbB-2.

5. CIRCULATING ErbB-2 AND ANTIBODIES

Despite ErbB-2 being a normal self-antigen and not yet shown to be mutated in human disease, significant antibody responses have been seen in the

presence of breast cancer. Disis showed titers greater than 1:100 in 20% of ErbB-2-expressing breast carcinoma patients (Disis *et al.*, 1997), with several of the patients having titers greater than 1:5,000. Tumors expressing ErbB-2 were found to release a soluble factor that corresponds to the extracellular domain of ErbB-2 (Langton *et al.*, 1991; Lin and Clinton, 1991). Circulating ErbB-2 itself, at serum levels greater than 120 fmol/ml, has been shown to be a negative prognostic factor for disease-free survival (Fehm *et al.*, 1997), poor prognosis in breast or ovarian cancer (Kandl *et al.*, 1994; Leitzel *et al.*, 1995; Mansour *et al.*, 1997; Meden *et al.*, 1997; Molina *et al.*, 1996; Willsher *et al.*, 1996), larger tumor size and nodal status (Fontana *et al.*, 1994; Krainer *et al.*, 1997), and decreased response to hormonal treatment (Leitzel *et al.*, 1995), but in many of the studies cited, was independent of the detection of ErbB-2 on the tumor specimens themselves by immunohistochemistry.

6. RESPONSE TO TREATMENT

The more aggressive phenotype of ErbB-2-overexpressing cells, both in clinical and laboratory settings, implied that the level of ErbB-2 is a predictive factor. Indeed, the reports reviewed next suggest some correlation with response to adjuvant hormonal and chemotherapy.

a. Hormonal Therapy

ErbB-2 overexpression has been linked to shorter disease-free survival and overall survival in ER positive patients treated with tamoxifen (Berns *et al.*, 1995; Borg *et al.*, 1994; Carlomagno *et al.*, 1996), but not in all studies (Elledge *et al.*, 1998). This concurs with basic research findings, in that tamoxifen induces ErbB-2 expression and enhances its signaling (Warri *et al.*, 1991, 1996). Recently, it has been shown that withdrawal of estrogen or treatment with tamoxifen leads to an increase in ErbB-2 expression, potentially due to intron 1 transcription factor binding sites, which mediate the transcriptional response to estrogens (Bates and Hurst, 1997).

b. Chemotherapy

As mentioned earlier, approximately 30% of patients with node negative disease are at risk of relapsing, thus there is a very practical need to identify tumors that might be more aggressive, suggesting the need for more intensive treatment protocols. Patients with node negative disease who were ErbB-2 positive were found to have a significantly decreased disease-free survival after chemotherapy (Allred *et al.*, 1992b). In a large study from the International (Ludwig) Breast Cancer Study Group Trial V, disease-free survival was greater for ErbB-2 negative patients, both node positive and node negative, who were treated with CMF (cyclophosphamide, methotrexate, 5-

fluorouracil) chemotherapy (Gusterson *et al.*, 1992). A prospective study from the Toronto Breast Cancer Study Group involving 580 patients identified ErbB-2 positivity as a significant negative prognostic factor in node negative patients, with the difference in disease-free survival accentuated among those receiving chemotherapy (Andrulis *et al.*, 1998). The poorer response to standard chemotherapy in patients overexpressing ErbB-2 is documented in node positive patients as well (Tetu and Brisson, 1994; Tsuda *et al.*, 1998). A case control study showed ErbB-2 positivity to predict poor disease-free survival after conservative surgery and radiotherapy without chemotherapy (Haffty *et al.*, 1996). The CALGB study comparing high-dose CAF [5-fluorouracil, doxorubicin (adriamycin), cyclophosphamide] chemotherapy with standard dose chemotherapy in women with node positive disease found that patients with ErbB-2 overexpression actually responded better than other patients after high-dose CAF treatment (Muss *et al.*, 1994). It is possible that the general improvement seen with the introduction of anthracyclines such as doxorubicin into standard and high-dose regimens such as CAF or AC may be explained by the finding that ErbB-2 is occasionally co-overexpressed with topoisomerase II α , and experiments *in vitro* show that the ErbB-2-topo II-overexpressing cells are relatively sensitive to inhibitors of the enzyme (Smith *et al.*, 1993). ErbB-2 as a prognostic factor has been studied in neoadjuvant therapy as well. In a study of neoadjuvant chemotherapy ErbB-2 overexpression was identified as the major prognostic factor correlated with disease-free survival and overall survival (MacGrogan *et al.*, 1996), although in a recent study of neoadjuvant CAF chemotherapy or radiotherapy, neither ErbB-2 nor p53 detection was of prognostic value (Rozan *et al.*, 1998). Last, in a study of patients with metastatic disease, ErbB-2 overexpression did not predict survival or response to chemotherapy (Niskanen *et al.*, 1997). These results suggest that it may be of value to document ErbB-2 status in the clinical trial setting, as an additional parameter that may influence choice of treatment protocol, particularly in early stage disease, where ErbB-2 overexpression may suggest including chemotherapy in the care of the node negative patient.

B. Gynecological Cancers

1. OVARIAN CANCER

Breast and ovarian cancer share responsiveness to steroid hormones, and incidence of the vast majority of tumors in the epithelial rather than the stromal component. In addition, these diseases appear to share etiologic factors: Women with one kind of tumor have an increased risk of developing the other type of tumor. Whereas normal ovarian tissue does not overexpress ErbB-

2 (Huettnner *et al.*, 1992; Wong *et al.*, 1995), 20–35% of borderline tumors (Eltabbakh *et al.*, 1997; Harlozinska *et al.*, 1997) and 30–50% of ovarian cancers express ErbB-2. About 60% of ovarian tumors may overexpress *erbB-2* by mRNA analysis (Huettnner *et al.*, 1992). In some, but not all studies (Medl *et al.*, 1995; Rubin *et al.*, 1993, 1994; Tanner *et al.*, 1996), ErbB-2 has been associated with advanced FIGO stage, worse prognosis, or decreased response to therapy (Berchuck *et al.*, 1990a; Harlozinska *et al.*, 1997; Meden *et al.*, 1994; Natali *et al.*, 1990; Slamon *et al.*, 1989). ErbB-2 expression by immunocytochemistry is higher in sporadic ovarian cancer than in the familial variety (Auranen *et al.*, 1997). Expression may also be higher in extra ovarian mullerian adenocarcinomas (Kowalski *et al.*, 1997). Unlike breast cancer, some ovarian tumor cell lines display rearranged *erbB-2* gene or variant transcripts (Hung *et al.*, 1992; King *et al.*, 1992), and more often than in breast cancer, overexpression may not be linked to gene amplification. According to one study, coexpression of ErbB-2 and p21/WAF1 correlates with shorter overall survival and disease-free survival (Katsaros *et al.*, 1995). High serum titers of an ErbB-2 fragment have been associated with shorter survival in ovarian cancer (Meden *et al.*, 1997).

2. VULVAR AND ENDOMETRIAL CANCER

Patients with stage I or II vulvar carcinoma are more likely to have nodal metastases if they overexpress ErbB-2 (Gordinier *et al.*, 1997). *erbB-2* Amplification is also associated with a more aggressive course in gestational trophoblastic disease (Bauer *et al.*, 1997). On the other hand, although overexpression was found in up to 48% of endometrial tumors, no overall prognostic significance was noted by some researchers (Backe *et al.*, 1997; Bell *et al.*, 1997; Gassel *et al.*, 1998). Nevertheless, others correlated more intense ErbB-2 staining with metastatic disease (Berchuck *et al.*, 1991), decreased survival (Hamel *et al.*, 1996; Kohlberger *et al.*, 1996), shorter disease-free survival by univariate analysis (Lukes *et al.*, 1994), and relation to deep myometrial invasion (Seki *et al.*, 1998). In stage I tumors treated by hysterectomy, overexpression of ErbB-2 has been correlated with decreased survival (Nazeer *et al.*, 1995). As in the case of ovarian and breast cancer, these results may be technique dependent. Gene amplification determined by using fluorescence *in situ* hybridization (FISH) did not correspond to protein expression in a series of endometrial tumors, but nevertheless amplification predicted poorer survival (Ribben *et al.*, 1997).

3. CERVICAL CANCER

ErbB-2 amplification was detected in 12–22% of stage II/III squamous cell cancers of the cervix (Kristensen *et al.*, 1996; Mitra *et al.*, 1994; Ndubisi

et al., 1997; Wong *et al.*, 1996). Increased ErbB-2 staining is seen in squamous metaplasia, raised condyloma, and carcinoma *in situ* (Berchuck *et al.*, 1990b). Staining for ErbB-1 was high, while staining for ErbB-2 decreased in cervical squamous neoplasms (Berchuck *et al.*, 1990b). Amplification of ErbB-2, H-ras, and c-myc was seen in high-grade cervical intraepithelial neoplasia (CIN3) but not in earlier stages (Pinion *et al.*, 1991). Cervical cancer patients with ErbB-2 overexpression showed significantly decreased 5-year survival in cohorts treated with radiation therapy (Nakano *et al.*, 1997, 1998), but decreased survival was not seen in all subgroups (Ndubisi *et al.*, 1997). In cervical glandular carcinoma *in situ* and adenocarcinoma, ErbB-2 overexpression is associated more frequently with human papilloma virus type 16 versus type 18 (Roland *et al.*, 1997).

C. Prostate Cancer

About 30% of prostate cancer stains positive for ErbB-2 (Kuhn *et al.*, 1993; Sadasivan *et al.*, 1993), whereas benign prostatic hypertrophy (BPH) is generally reported as being negative (Kuhn *et al.*, 1993; Sadasivan *et al.*, 1993). Some studies that showed strong positive staining for ErbB-2 in malignant tissue have demonstrated positive immunostaining for ErbB-2 in BPH as well (Giri *et al.*, 1993; Gu *et al.*, 1996). In fact, discrimination between membrane or cytoplasmic staining may yield an even stronger difference between benign and malignant tissue. A retrospective study showed ErbB-2 staining by immunocytochemistry to correlate with disease progression in node negative patients (Veltri *et al.*, 1994). As with endometrial tumors, the recent use of FISH for analysis of prostate carcinoma has proven itself to be more sensitive, showing about 40% positivity versus 29% by IHC, and positivity was significantly associated with higher Gleason grade, DNA ploidy, and disease-free survival (Ross *et al.*, 1997a,b).

D. Gastrointestinal Cancer

I. COLON CANCER

While normal colonic mucosa is mostly negative for ErbB-2, the levels of this antigen increase with Dukes stage in colon carcinoma and show significant correlation with relapse-free and postoperative survival period (Kapitanovic *et al.*, 1997). Consistent with a correlation with poor prognosis, colon cancers that metastasized to the liver have higher levels of ErbB-2 than those that do not (Yang *et al.*, 1997). ErbB-2 overexpression was found in about 50% of early colon carcinomas, particularly those with-

out an adenomatous component (Caruso and Valentini, 1996; Shirai *et al.*, 1995), and colon cancer patients with lymph node metastases were reported to have higher ErbB-2 levels (Saeki *et al.*, 1995). Interestingly, significant trends for coexpression of ErbB-1 and either TGF- α or amphiregulin were detected in this latter study, suggesting that the two ligands of ErbB-1 may play an important role in the development of colorectal carcinomas through an autocrine mechanism. Lastly, circulating serum levels of ErbB-2 correlated with poor prognosis in colorectal carcinoma (Vogel *et al.*, 1996).

2. ESOPHAGEAL CANCER

One study showed 43% ErbB-2 positivity in esophageal tumors, and association with a better prognosis to neoadjuvant therapy (Duhaylongsod *et al.*, 1995). In Barrett's adenocarcinoma expression correlates with decreased patient survival (Flejou *et al.*, 1994).

3. GASTRIC CANCER

Overexpression of ErbB-2 in gastric cancer is common (Kim *et al.*, 1993; Ooi *et al.*, 1998), particularly in advanced stage and intestinal subtype (Wu *et al.*, 1998) and may be associated with worse prognosis (Amadori *et al.*, 1997; Yonemura *et al.*, 1998).

4. PANCREATIC CANCER

ErbB-2 is frequently overexpressed in pancreatic adenocarcinomas, specifically in well- or moderately differentiated glandular areas of the tumor, and is decreased in the poorly differentiated areas (Dugan *et al.*, 1997; Yamanka *et al.*, 1993b). Overexpression was linked with worse prognosis in ampulla of Vater tumors (Vaidya *et al.*, 1996a,b).

E. Lung Cancer

A significant additive effect between p53 and ErbB-2 in predicting poor prognosis was found in stage I NSCLC (Harpole *et al.*, 1995), but not in a larger study (Pastorino *et al.*, 1997). ErbB-2 expression has an inverse correlation with angiogenesis in NSCLC (Giatromanolaki *et al.*, 1996). The combination of K-ras mutation by PCR with ErbB-2 expression by immunocytochemistry was a poor prognostic factor in lung NSCLC (Nemunaitis *et al.*, 1998).

F. Head and Neck Cancer

Overexpression of ErbB-2 is associated with a decreased disease-free survival in intestinal-type adenocarcinoma of the paranasal sinuses (Gallo *et al.*, 1998), palatal salivary gland neoplasms (Giannoni *et al.*, 1995), and mucoepidermoid carcinoma of the salivary gland (Press *et al.*, 1994).

G. Kidney Cancer

An inverse relationship between ErbB-1 and ErbB-2 levels in renal cell carcinoma has been shown (Weidner *et al.*, 1990). ErbB-2 overexpression was noted with increased frequency in certain renal cystic disorders as well as in neoplasms (Herrera, 1991). Combined high expression of ErbB-1 and ErbB-2 was correlated with metastatic disease (Stumm *et al.*, 1996).

H. Bladder Cancer

A relationship between transitional cell cancer of the bladder and ErbB-2 expression was first noted by Zhau *et al.* (1990) who found that about 70% of tumors, and none of the normal tissue specimens, overexpress ErbB-2 by immunohistochemistry and Western blotting. Overexpression of erbB-2 has been correlated with grade and survival (Korkolopoulou *et al.*, 1997; Lipponen *et al.*, 1991; X. H. Zhang *et al.*, 1997), and is infrequent in superficial tumors (Tetu *et al.*, 1996). The presence of disease is frequently diagnosed through bladder washings, and the presence of ErbB-2 in the cytological specimen is found only in tumors of higher grade (Lonn *et al.*, 1993). Interestingly, in a large recent prospective study, presence of ErbB-1 or ErbB-2 in grade 3 tumors predicted for less invasive disease (Vollmer *et al.*, 1998).

I. Brain Tumors

In astrocytomas, ErbB-2 tends to correlate with higher grade histology (Bernstein *et al.*, 1993; Schwechheimer *et al.*, 1994). On the other hand, in meningiomas, expression of ErbB-2 is higher in the typical forms, whereas expression is lost in progression to atypical forms; the inverse is true for p53 (Chozick *et al.*, 1996). PCR analysis of CNS fluid can detect *erbB-2* sequences from breast cancer metastatic to brain (Rhodes *et al.*, 1994). In neuroblastoma, ErbB-2, but not ErbB-1, predicted significantly shorter patient

survival, and the combination of ErbB-2 with p53 positivity was especially strong as a prognostic indicator (Layfield *et al.*, 1995).

III. HOW DOES ErbB-2 INDUCE CANCER?

A. *In Vitro* Transforming Potential of ErbB-2

How ErbB-2 causes cancer is a question that has been repeatedly asked—and the answers reevaluated—during the past 15 years. Its role in cellular transformation, insinuated by its abundance in a wide range of human tumors, is supported by a few but solid lines of evidence that therapies directed against this receptor can indeed impede tumor growth. Although no analogous point mutation, to that of the transforming rat *neu*, was found in the human gene, site-directed mutagenesis confirmed that a similar change can activate human *erbB-2* as an oncogene (Akiyama *et al.*, 1991). When overexpressed in mouse fibroblasts, the human gene conferred a transformed phenotype *in vitro* and tumorigenesis *in vivo* (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987), indicating that a threshold level of overexpression is crucial for its oncogenic potential. In accordance, manipulating ectopic expression of ErbB-2 by tetracycline-induced abrogation reversed the transformed phenotype of the cells and their ability to establish tumors (Baasner *et al.*, 1996). Moreover, tissue-specific induction of the human, unmutated transgene promoted the appearance of mouse mammary adenocarcinomas with a tendency to metastasize (Suda *et al.*, 1990). Tumor progression by ErbB-2 overexpression was attributed to aberrant activation of the tyrosine kinase (Lonardo *et al.*, 1990; Pierce *et al.*, 1991) and indeed it has been demonstrated that this activity is essential for transformation (Weiner *et al.*, 1989a). Consequently the removal of ErbB-2 from the cell surface of breast cancer cells (Beerli *et al.*, 1994) reduces mitogenicity and correlates this effect with a downstream decrease in growth factor-induced signaling (Graus-Porta *et al.*, 1995; Karunakaran *et al.*, 1996). Cellular transformation by ErbB-2 is, therefore, most probably linked to its elevated expression at the cell surface where it can initiate a hypermitogenic intracellular signal.

B. ErbB Activating Ligands

Residing in cells of epithelial origin, ErbB receptors can be nourished with binding proteins either via an autocrine secretory loop or with molecules produced by adjacent tissues. Stromal cells in the vicinity of propagating tumors serve as the prime source for ErbB-activating ligands, whereas the ex-

tracellular matrix surrounding them is a reservoir that can increase local ligand concentrations (see Fig. 2).

EGF was the first identified and is the prototype for ligands capable of binding ErbB proteins. These have been shown to harbor the capacity to activate the receptors within a common structural motif of 45–55 amino acids, called the EGF domain. Three covalently held loops, formed by six cysteine residues that are typically spaced within this motif, are crucial for receptor recognition and, together with a critical arginine residue and several glycines, are shared by all ligands (Groenen *et al.*, 1994). All mammalian ErbB ligands are derived from transmembrane precursors and give rise to the mature soluble protein by specific cleavage. Similar to EGF, the transforming growth factor α (TGF- α) (Marquardt *et al.*, 1984), amphiregulin (Shoyab *et al.*, 1988), betacellulin (Sasada *et al.*, 1993), epiregulin (Toyoda *et al.*, 1995), and the heparin-binding EGF (HB-EGF) (Higashiyama *et al.*, 1991) can all induce activation and phosphorylation of ErbB-1, but none are able to stimulate homodimeric complexes of ErbB-2. Correlation between these ligands and

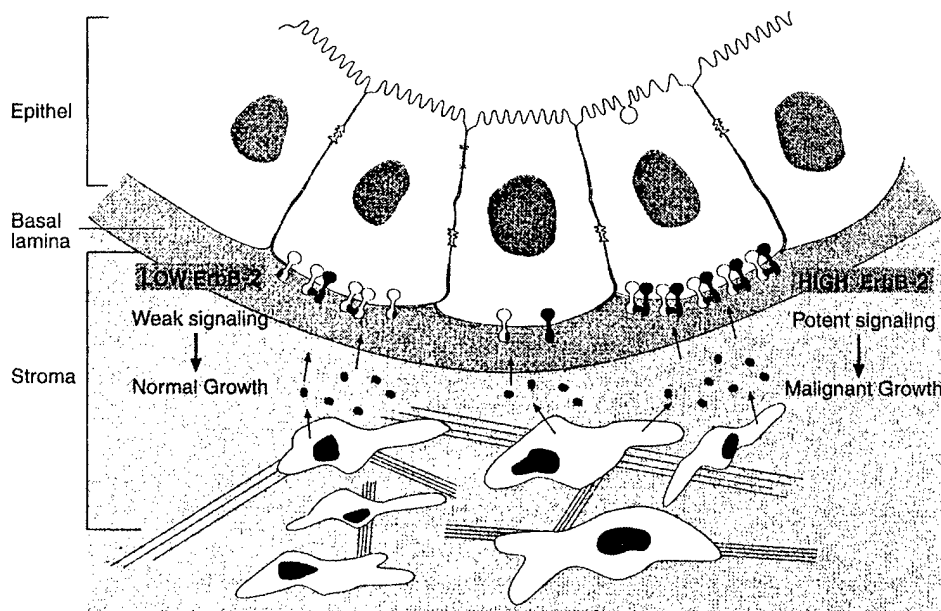


Fig. 2 Epithelial-mesenchymal interactions in the promotion of cancer. Cells of mesenchymal origin, embedded within the stroma, synthesize and secrete EGF-like growth factors. Diffusing through the basal lamina, surrounding glandular structures, the polypeptide ligands encounter binding receptors exposed on the surface of epithelial cells. The receptor repertoire of the cell can determine the biological response achieved by such interactions. Accordingly, high expression of ErbB-2 can contribute to the amplification of growth factor signaling, driving the cells toward a malignant phenotype. Moreover, extracellular matrix can serve as a ligand reservoir, increasing its local concentrations available for receptor activation.

cancer has been most convincingly demonstrated for TGF- α . The expression of this ligand, accompanied by its binding receptor ErbB-1, correlates with poor prognosis for several gastrointestinal (Ihara *et al.*, 1993), lung (Tateshi *et al.*, 1990), and ovarian (Kohler *et al.*, 1989) malignancies. In a similar manner, the coexpression of ErbB-1 with either EGF or TGF- α was reported in 38% of pancreatic tumors and correlated with both tumor size and a reduction in patient survival (Yamanaka *et al.*, 1993a). Enhanced synthesis of EGF has been reported in several tumors, including lung and ovarian tumors and the levels of both growth factors were found to be elevated in the urine of glioma patients. Another ligand, HB-EGF, may act as a stromal mediator of prostate cancer, being synthesized by interstitial and vascular smooth muscle cells and capable of efficiently stimulating cell growth (Freeman *et al.*, 1998).

Neuregulins (NRGs) comprise an additional group of ErbB activating proteins, demonstrating a specificity of binding toward ErbB-3 and ErbB-4 (Burden and Yarden, 1997). NRG1, which was initially identified as an ErbB-2 phosphorylating activity (Falls *et al.*, 1993; Holmes *et al.*, 1992; Mabrouk *et al.*, 1996; Marchionni *et al.*, 1993; Peles *et al.*, 1992; Wen *et al.*, 1992), inhibits terminal differentiation and induces adenocarcinomas when targeted to the mammary gland of transgenic mice (Krane and Leder, 1996), suggesting involvement in tumor-propagating processes mediated by ErbB receptors. The exact mode of action by which NRG exerts its effect on epithelia is not simple, as exemplified by its counteracting effects on cells of the mammary gland. Essentially, synthesis of the ligand by the connective tissue, primarily the fat pad, surrounding ductal and alveolar structures, is upregulated at pregnancy and is able to promote lobular-alveolar budding and milk production (Yang *et al.*, 1995). Several breast cancer cell lines differentiate, in accordance with this phenomenon, to produce milk proteins and lipids (Bacus *et al.*, 1992a, 1993). However, others undergo enhanced proliferation in response to NRG stimulation (Lewis *et al.*, 1996). A variety of biological observations led to the identification of splice variants of the NRG1 gene revealing their pleiotropic mode of action. Among these are the glial growth factor (GGF), which induces Schwann cell proliferation (Marchionni *et al.*, 1993), and acetylcholine receptor inducing activity (ARIA) functioning at the neuromuscular junction (Falls *et al.*, 1993). A related group of molecules, termed NRG2, binds both ErbB-3 and ErbB-4 (Carraway *et al.*, 1997; H. Chang *et al.*, 1997), and a third molecule, NRG3, exclusively binds to ErbB-4 (D. Zhang *et al.*, 1997).

C. Does ErbB-2 Have a Ligand of Its Own?

Intuitively, a transforming activity transmitted by overexpressed levels of ErbB-2 could be evoked by a direct ligand. Such a binding protein would in-

duce receptor homodimerization, cumulatively resulting in a high mitogenic signal. Several candidate ligands suggested to fulfill this function have been isolated. Among these are NAF (neu activating factor) isolated from a transformed human T-cell line (Samanta *et al.*, 1994), p75 purified from human mammary carcinoma cells (Lupu *et al.*, 1992), and a surface proteoglycan from a rat adenocarcinoma (Wu *et al.*, 1994). However, although capable of stimulating ErbB-2 phosphorylation, the specificity and function of these putative ligand molecules are not well established, maintaining ErbB-2's orphanhood.

Reinforcing the nonexistence of a direct ErbB-2 ligand is the identification of three EGF-like ligands encoded by poxviruses: the vaccinia virus growth factor (VGF) (Brown *et al.*, 1985; Stroobant *et al.*, 1985), the Shope sarcoma virus growth factor (SGF) (Chang *et al.*, 1987), and the Myxoma virus growth factor (MGF) (Upton *et al.*, 1987). Apparently, these ligands harness the proliferation-inducing activity of the receptors for the enhancement of their virulence (McFadden *et al.*, 1996). Synthetic analogs of these three viral ligands revealed a specific pattern of binding to ErbB proteins: Whereas VGF is similar to EGF in its receptor specificity, and SFGF is a pan-ErbB ligand, MGF emerges as a factor that binds exclusively to heterodimeric ErbB-2/ErbB-3 complexes, but not to either of the corresponding homodimers (Tzahar *et al.*, 1998). Likewise, neither VGF nor SFGF can recognize ErbB-2 alone, but both can recruit it into heterodimers, suggesting that no high-affinity ligand of ErbB-2, of either viral or mammalian origin, may exist (Tzahar *et al.*, 1998; Klapper *et al.*, 1999).

D. Ligand-Independent Receptor Dimerization

An alternative to ligand-induced activation of the ErbB-2 protein is a conformational change driven by dimer formation in a ligand-independent manner. Conformational energy analysis of the transmembrane domain of the receptor predicted that the mutated transforming Neu, presenting a helical form of the sequence, should have a higher tendency toward dimer formation than that of the wild-type protein (Brandt Rauf *et al.*, 1990). Indeed, covalent cross-linking could detect dimers of the transforming protein, whereas the normal receptor appeared only as a monomer (Stern *et al.*, 1988; Weiner *et al.*, 1989b). Apparently the glutamic acid residue, substituting the normal valine at position 664 in the transmembrane portion of the wild-type Neu, is strongly hydrogen bonded, suggesting that direct interactions of this residue underlie the propensity to form dimers (Smith *et al.*, 1996). Dimer formation between molecules of transforming Neu is indeed essential as demonstrated by the ability of a cytoplasmic portion deleted oncoprotein to inhibit transformation and tumorigenicity of Neu-expressing fibroblasts and

rat-derived neuroglioblastomas (Qian *et al.*, 1996). This dominant-negative effect of the dimerizing partner is attributed to the lack of kinase activity and can be reproduced by mutating the ATP-binding site of the receptor, indicating that the proliferative effect is dependent on an intact signaling cascade triggered by receptor transphosphorylation (Schlegel *et al.*, 1997). The quality and not only the level of receptor phosphorylation seems to be important because receptor dimerization induced by a foreign transmembrane sequence elevated phosphorylation on tyrosine residues, but lacked the ability to cause transformation *in vitro* (Burke *et al.*, 1997). Interestingly, mammary tumors induced by a transgenic wild-type Neu arise due to somatic activating mutations within the extracellular part of the receptors. These mutations, occurring at conserved cysteine residues, promote constitutive dimerization via disulfide bonds, resulting in a transforming activity (Siegel and Muller, 1996).

The question of transforming mechanisms remains, however, unanswered, because none of the suggested dimerization-driving mutations has been identified in human cancer. Moreover, homodimers of the wild-type ErbB-2, induced by monoclonal antibodies (Pinkas-Kramarski *et al.*, 1996b) or a point mutation (Campion *et al.*, 1993), imply that the activity of the homodimeric ErbB-2 complexes is relatively weak, suggesting that constitutive homodimerization arising due to high receptor expression would not necessarily lead to transformation. In support of this scenario, transfection of oncogenic Neu conferred ligand-independent transformation only in the presence of ErbB-1, ErbB-3, or ErbB-4 (Cohen *et al.*, 1996b). Possibly, heterodimer formation, more than the formation of ErbB-2 homodimers, is linked to a transforming signal.

IV. EVOLUTIONARY AND DEVELOPMENTAL ASPECTS OF THE MULTIPLICITY OF ErbB PROTEINS

Signaling via ErbB receptor tyrosine kinases matured to the module known in mammals following a paradigm of increased complexity, enabling interactions in a tissue-specific manner as well as a high degree of fine tuning and regulation. ErbB signaling was originally handled by a single family member as represented in *Caenorhabditis elegans* by the *let-23* gene encoding a primordial EGF receptor (Aroian *et al.*, 1990). ErbB structural motifs, as well as the amino acid sequence of the cytoplasmic tyrosine kinase domain, were inherited through evolution as was the structure of a single Let-23 binding protein, the Lin-3 ligand, bearing a typical EGF-like domain (Hill and Sternberg, 1992). The relative simplicity of ErbB signaling in *C. elegans* enabled the characterization of its central role in development as reflected in vulval

induction essential for nematode reproduction. Gonadal secretion of the Lin-3 protein exposes the adjacent vulva precursor Let-23-expressing cells to a ligand gradient, resulting in a series of divisions and morphogenetic changes leading to vulval generation (Sternberg and Horvitz, 1991). Cell fate determination by Let-23 activation was also demonstrated in neuroectoblasts of *C. elegans*, however, unlike vulval induction, differentiation is uncoupled to cell division (Sternberg *et al.*, 1995), indicating a versatility of action overriding the expected limitations of a single-receptor-ligand interaction.

Proceeding several steps higher in evolution, to the fruit fly *Drosophila*, reveals a complexity achieved by ligand multiplicity represented by four members: *gurken*, *spitz*, *vein*, and *argos* (Neuman-Silberberg and Schupbach, 1993; Rutledge *et al.*, 1992; Schnepp *et al.*, 1996; Schweitzer *et al.*, 1995), all apparently capable of binding a single receptor homolog, the *Drosophila* EGF receptor (DER) protein (Livneh *et al.*, 1985). Isolation of mutant alleles of the *Drosophila* receptor revealed the central role of the DER signaling pathway in the development of multiple tissues and organs spanning a temporal range of processes (Clifford and Schupbach, 1994; Shilo and Raz, 1991). As in lower and higher organisms, DER is widely expressed in embryos, assigning its regional activation to spatial restriction of the ligands (Stein and Stevens, 1991).

Expanding the complexity even further, evolutionary progress applied repeated duplications of genes encoding the ErbB receptors, as well as their EGF-like ligands, achieving the wide repertoire known in mammals. ErbB-dependent developmental patterns supplied valuable evidence that the availability of several ligand-receptor combinations evolved to serve an interplay of receptor interactions rather than to expand parallel nonoverlapping pathways. The first family member that was successfully targeted and thereby expression eliminated was the *erbB-1* gene. Absence of ErbB-1 expression is lethal, death occurring at a variety of developmental phases depending on animal genetic background, as a result of major defects in the assembly of epithelia (Miettinen *et al.*, 1995; Sibia and Wagner, 1995; Threadgill *et al.*, 1995). Ligand multiplicity predicted that disruption of a single gene would result in a milder phenotype than that arising from receptor knockout. Indeed, mice homozygous for a disrupted TGF- α gene displayed only part of the abnormalities of *erbB-1*^{-/-} mice, the most eminent being aberrant eye development and waviness of whiskers (Luetkeke *et al.*, 1993; Mann *et al.*, 1993). A different ligand, HB-EGF, seems to govern yet another role of ErbB-1 in development, enabling blastocyte attachment to the uterus (Das *et al.*, 1994).

Signaling by ErbB-3 and ErbB-4 inflicts an additional level of complexity by applying a wide but shared variety of direct ligands to both receptors. Genetic evidence implies that signaling by neuregulins is essential for epithelial organs, for effectors of the nervous system such as Schwann cells and neu-

romuscular junctions, and for muscle cells of the heart's trabeculae (Meyer and Birchmeier, 1995). Mouse embryos lacking ErbB-4 die of heart malfunction caused by an undifferentiated ventricle, manifested in the absence of myocardial extensions (Gassmann *et al.*, 1995). This phenotype is shared by NRG mutants specifically targeted at immunoglobulin-like (Ig-like) domain containing isoforms, indicating that ligand abundance does not confer redundancy in biological effects (Kramer *et al.*, 1996). This important feature of multiplicity is further emphasized by the requirement for Ig-NRGs that have a glycosylation domain (type I), as opposed to NRGs without a glycosylation domain (type II), playing an important role in the early development of Schwann cells, but not in heart differentiation (Meyer *et al.*, 1997).

Remarkably, the phenotype of *erbB-2*^{-/-} mice shares characteristics with both NRG and ErbB-4 knockout animals (Lee *et al.*, 1995). This phenotypic similarity to both *NRG*^{-/-} and *erbB-4*^{-/-} mice implies the three genes to be of close relevance in development and essential for the activation of an overlapping pathway. Likewise, targeted mutations to the ErbB-3 receptor cause severe neuropathies (Riethmacher *et al.*, 1997), the earliest observed, namely, absence of neurons deriving from hindbrain, being similar to an *erbB-2*^{-/-} phenotype. Such an overlap in activities suggests that ErbB-2 cooperates with ErbB-3 and ErbB-4 in the mediation of signals induced by NRGs. As discussed later, this cooperation appears essential for ErbB-2-mediated transformation of epithelial and other cell types.

V. THE ErbB SIGNALING NETWORK

A. Evidence for Inter-Receptor Interactions

Functional interactions between ErbB proteins were first suggested by the observation that ErbB-2 is a substrate of the ligand-activated ErbB-1 (King *et al.*, 1988; Stern *et al.*, 1986). This was later shown to correlate with a physical association of the two proteins leading to the formation of heterodimeric complexes and strictly dependent on the binding of a ligand (Goldman *et al.*, 1990; Wada *et al.*, 1990b). Molecular modeling of the kinase domains of both receptors implied that heterodimers are energetically favored over homodimers (Murali *et al.*, 1996). In line with this prediction, all six possible heterodimeric complexes of ErbB receptors can be observed (Tzahar *et al.*, 1996). The most remarkable variation achieved by this phenomenon is due to the impaired catalytic activity of ErbB-3 requiring a partner with an active kinase to promote its signaling (Guy *et al.*, 1994). Assessing the incidence of dimer formation reveals that an ErbB receptor bound by its direct

ligand will preferentially recruit ErbB-2 as a heterodimerizing partner (Graus Porta *et al.*, 1997; Tzahar *et al.*, 1996). Moreover, EGF and NRG receptors compete with each other for the interaction with the ErbB-2 receptor (Karunagaran *et al.*, 1995), implying an advantage of ErbB-2 as a signaling subunit. Complying with its emerging role as a favored surrogate receptor, the expression of ErbB-2 is the most expanded amongst all four family members (Pinkas-Kramarski *et al.*, 1997).

B. Transforming Ability of Heterodimers

The bias toward the formation of ErbB-2-containing heterodimers could have evolved via superiority of their signaling. Indeed, signals generated by activated ErbB-1/ErbB-2 heterodimers lead to an enhanced proliferative response to EGF (Wada *et al.*, 1990a), resulting in greater normal and tumorigenic cell growth in comparison to ErbB-1 homodimers (Kokai *et al.*, 1989). Accordingly, transgenic expression of both Neu and TGF- α in the mammary epithelium appears synergistic in the promotion of multifocal mammary tumors arising after a significantly shorter latency period than either parental strain alone (Muller *et al.*, 1996).

Importantly, none of the ErbB receptors alone could cause growth in soft agar or tumorigenicity in animals, transformation occurring only when ErbB-2 was expressed with one or more of its sibling receptors (Cohen *et al.*, 1996b). This heterodimer-dependent transformability of the complexes was further demonstrated by its inhibition with antibodies against either participating receptor (Wada *et al.*, 1990a), as well as by reducing cell proliferation with heterodimer-destabilizing monoclonal antibodies (Klapper *et al.*, 1997).

Both ErbB-3 and ErbB-4 show a mitogenic superiority and promote cellular transformation (Alimandi *et al.*, 1995; Wallasch *et al.*, 1995; Zhang *et al.*, 1996) in a ligand-dependent manner, when co-introduced with ErbB2. Most significant is the reconstitution of the ErbB2/ErbB-3 heterodimer. Induced by NRGs, this complex accommodates the highest signaling activity among all receptor combinations (Pinkas-Kramarski *et al.*, 1996b; Riese *et al.*, 1995) as well as being the predominant ligand-binding moiety in several human adenocarcinomas (Chen *et al.*, 1996). The functional linkage between the increased mitogenicity of ErbB-2-containing dimers and its high expression in cancer could reside, on the one hand, in its high basal activity (Lonardo *et al.*, 1990) and, on the other, in a propensity to form dimers (Weiner *et al.*, 1989a). The latter, reminiscent of the transforming rodent Neu, appears crucial to understanding the role of ErbB-2 in signal transduction and in cancer: Although not a direct receptor for EGF or NRG,

ErbB-2 can decrease the rate of dissociation of these ligands from their direct receptors (Karunagaran *et al.*, 1996). This results in significant prolongation of downstream signaling to the mitogen-activated protein kinase (MAPK) pathways and remarkable signal amplification. Thus, by forming heterodimers with other ErbB proteins, ErbB-2 may function as an amplifier of stroma-derived mitogenic signals (Fig. 3). As discussed later, this capability is enhanced by two factors: First, the propensity of ErbB-2 to undergo endocytosis is low compared to that of ErbB-1, and second, ErbB-2-containing heterodimers are endowed with an unexpectedly wide specificity to various ErbB ligands.

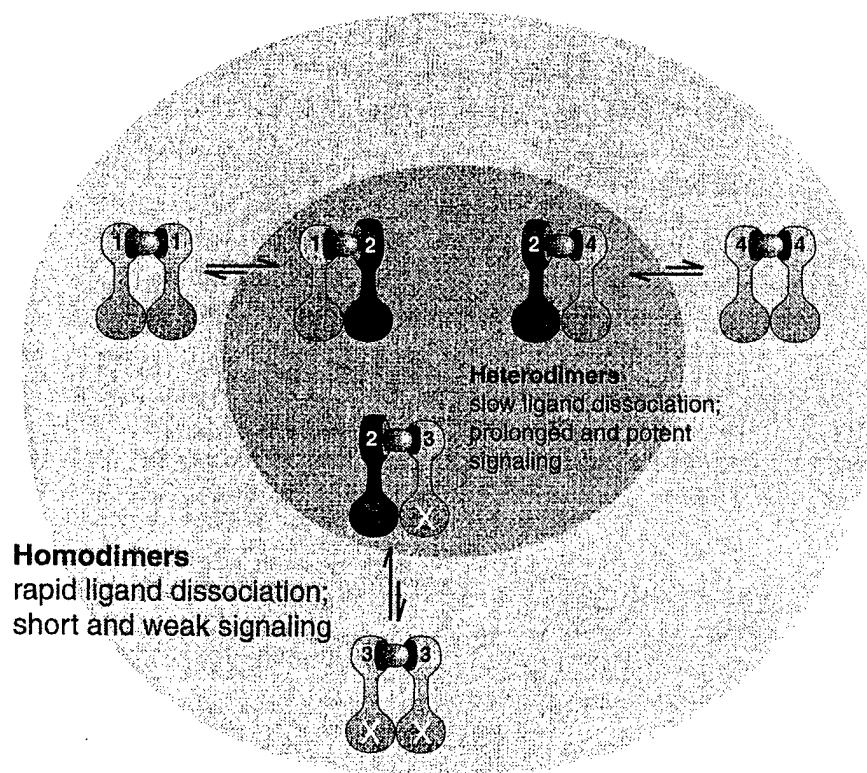


Fig. 3 Signaling superiority of ErbB-2-containing heterodimers. ErbB receptor dimers comprise the ligand-binding and signal-promoting complexes. ErbB-2 ("2") is a superior heterodimerizing partner in comparison to sibling receptors. Superiority is attributed to a hierarchy of recognition by which ErbB-2 is preferred to other members. Furthermore, complexes comprised of ErbB-2 molecules show high stability in comparison to non-ErbB-2-containing dimers (as expressed by the balance between arrows). Dimer stability is reflected in the affinity of EGF-like ligands to their binding moieties, being higher in ErbB-2 containing heterodimers than in homodimers. High affinity is the result of a decrease in ligand dissociation rate and the outcome is a strong and prolonged signal.

C. Ligand-Bivalency Selects Dimer Participants

The ability of ErbB-2 to increase ErbB signaling in a ligand-dependent fashion, despite its lack of a direct binding protein of its own, addresses a fundamental question of dimerization-driving forces. The mechanism of receptor dimerization is best understood in the case of the human growth hormone receptor (Wells, 1996), in which the ligand uses two different sites to sequentially bind two receptor molecules. A similar model is suggested for ErbB receptors, originally relying on the duplicated structure of ErbB-1's extracellular domain (Gullick, 1994), and recently supported by evidence of bivalent recognition of EGF-like ligands (Tzahar *et al.*, 1997). According to this model, encouraged by NMR studies (Jacobsen *et al.*, 1996), NRG1 bears two distinct receptor binding sites, an N-terminal high-affinity site and a low-affinity/broad-specificity site located at the C terminus (Barbacci *et al.*, 1995; Tzahar *et al.*, 1997). High-affinity binding of the ligand to its primary receptor demands a stringent fit, whereas the low-affinity site allows more flexible pairing. Furthermore, derivatives of EGF indicated that the N-terminal tail of this ligand binds to the N-terminal subdomain of its receptor, whereas the C terminus of EGF juxtaposes to subdomain III of ErbB-1 (Summerfield *et al.*, 1996), pinpointing this domain as a possible low-affinity broad-specificity binding pocket. It is suggested that a functionally analogous portion exists within the ErbB-2 receptor, selecting it as a preferred coreceptor. Supporting this is the cooperative binding of both EGF (Wada *et al.*, 1990b) and NDF (Peles *et al.*, 1993; Sliwkowski *et al.*, 1994; Tzahar *et al.*, 1996) to cells co-overexpressing a primary receptor together with ErbB-2, implying its direct low-affinity binding to the ligands. In accordance, biophysical assays reported that ErbB-2 binds EGF-like ligands at an affinity ranging in micromolars as opposed to the nanomolar range affinity of the ligands to their primary respective receptor (Horan *et al.*, 1995; Tzahar *et al.*, 1997). In addition, monoclonal antibodies directed against the putative ligand binding site of ErbB-2 can accelerate dissociation of EGF-like ligands, thereby blocking the formation of ErbB-2-containing dimers (Kalpper *et al.*, 1997). Cumulatively these independent studies reason that ErbB-2, capable of directly recognizing bivalent ligands, has no ligand of its own and has evolved to function as a shared signaling subunit of ErbB receptor complexes, analogous to the gp120 shared subunit of lymphokine receptors.

D. Extending the Variation of Signaling Complexes by Diversification of Ligand Recognition

According to the model implying bivalence of ErbB binding ligands, each EGF-like domain can select its own unique set of preferred receptor dimers,

suggesting that ligand multiplicity is segregated not only by differential expression but also by distinct recognition. Indeed, two ErbB-1 activating ligands, EGF and TGF- α , have been shown to bind their receptor at nonoverlapping sites (Katsuura and Tanaka, 1989; Richter *et al.*, 1992). This is extended to variations in the recruitment of dimeric partners as demonstrated for two isoforms of NRG1, both able to stabilize ErbB-2/ErbB-3 heterodimers, whereas only NRG1- β can promote ErbB-1/ErbB-3 heterodimers (Pinkas-Karmarski *et al.*, 1996a). A similar diversity in complex formation is suggested for ErbB-1 ligands different in transactivation of the various ErbB receptors (Beerli and Hynes, 1996). Complex variation is further increased by the dual specificity of betacellulin (Riese *et al.*, 1996) and HB-EGF (Elenius *et al.*, 1997), which bind both ErbB-1 and ErbB-4. In addition to its ability to stabilize ligand-bound complexes, and thereby prolong signal transduction, the superiority of ErbB-2 as a dimerizing partner is enhanced by its ability to expand specificity of ligand-receptor recognition. Epiregulin, a broad specificity ligand that preferentially binds ErbB-4 (Komurasaki *et al.*, 1997), gains ErbB-1 activation and augments ErbB-4 activation in the presence of ErbB-2 (Riese *et al.*, 1998; Shelly *et al.*, 1998). Cross-specificity, conferred by ErbB-2, is further demonstrated by the ability of high concentrations of EGF and betacellulin to promote ErbB-2/ErbB-3 dimers (Alimandi *et al.*, 1997; Pinkas-Karmarski *et al.*, 1998) and the ability of EGF, TGF- α , and HB-EGF to signal through ErbB-2/ErbB-4 complexes (Shelly *et al.*, 1998; Wang *et al.*, 1998).

E. Intracellular Signaling

The diversity of dimer formation could be attractively rationalized by the possibility that every ligand-bireceptor complex recruits a unique set of signaling proteins activating a distinct pathway. Indeed, a large number of cytoplasmic proteins, containing phosphotyrosine binding motifs, engage the activated ErbB dimers. However, in contrast to the dogma underlying diversity, many of these proteins overlap, interacting with most if not all dimeric species (Alroy and Yarden, 1997). These include effectors such as Shc (Pellicci *et al.*, 1992; Segatto *et al.*, 1993), Grb-2 (Buday and Downward, 1993), and Src (Anderson *et al.*, 1990; Luttrell *et al.*, 1994). Other substrates showing some specificity are Cbl, a protooncogenic adaptor that is recruited by all ErbB-1 containing dimers (Levkowitz *et al.*, 1996); phospholipase C γ (PLC γ), which associates with ErbB-1 and ErbB-2 (Cohen *et al.*, 1996a; Peles *et al.*, 1991), but not with ErbB-3 and ErbB-4; and phosphatidylinositol 3'-kinase (PI3K), which shows a preference toward ErbB-3 (Stoltoff *et al.*, 1996). Additionally, certain phosphorylated docking sites have permissive recognition enabling the binding of more than one adaptor via a hierarchy

that is characteristic of each receptor. Such is the preferential selectivity of ErbB-3 toward Grb-7 upon Grb-2 (Fiddes *et al.*, 1998), suggesting the interaction to be dependent on the cellular milieu.

Despite the wide availability of transmitting molecules, mammalian ErbBs conserve the signaling pattern observed in invertebrates that utilizes the Ras-Raf-MAP-kinase cascade as its major route (Brunner *et al.*, 1994; Hsu and Perrimon, 1994). It seems that receptor identity determines both the intensity and the kinetics of MAPK activation, increasing from homodimers to heterodimers and more so in heterodimers containing ErbB-2 (Graus-Porta *et al.*, 1995; Karunagaran *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996b). The fine tuning of this cascade most probably governs the decision of differentiation or proliferation on growth factor stimulation (Marshall, 1995). Accordingly, NRG1 stimulation of cancer cells can cause proliferation or differentiation, depending on receptor expression (Bacus *et al.*, 1992b; Daly *et al.*, 1997), both effects correlating with the activation of MAPK (Grasso *et al.*, 1997; Sepp Lorenzino *et al.*, 1996). Likewise, dominant-negative inhibition of the MAPK-activating pathway abrogated transcriptional stimulation by NRG in differentiating myotubes (Altiook *et al.*, 1997; Si *et al.*, 1996).

Regardless of the biological outcome, it seems that the response triggered by ErbB-specific growth factors follows a pattern of signals that spreads outward from the activated receptor and passes down a number of parallel pathways before converging onto a specific set of signaling molecules. Theoretically this can stabilize cell signaling pathways against transient fluctuations in the concentrations of cell signaling molecules. The emergence of ErbB-2 as a favored signaling subunit of ErbB receptors highlights this concept even further due to its apparent strong coupling to MAPK (Ben-Levy *et al.*, 1994). ErbB-2 couples a deleted ErbB-1, lacking its phosphotyrosine residues, to the Ras signaling pathway, recovering the DNA synthesis characteristic of the wild-type ErbB-1 (Sasaoka *et al.*, 1996). Furthermore, a kinase-defective Neu abrogates motigenicity and transformation by ErbB-1, without causing its uncoupling to typical signaling molecules such as PLC γ , the ras-GTPase activating protein (Ras-GAP), and Shc, suggesting additional pathways underlying ErbB-2 superiority (Dougal *et al.*, 1996). In such a case the ErbB-2-dominated network will respond correctly even if changes to the network occur or the input is incomplete. Parallel network-pathways, originated to ensure correct signaling, could underlie pathological hypersignaling, leading to unregulated cell proliferation. Interestingly and in agreement with this possibility, two ErbB-2 binding proteins, Grb-2 and Grb-7, although known to interact with the same receptor residue, do not compete with each other in breast cancer cells. Apparently, a common co-overexpression of Grb-7 and ErbB-2 is correlated with increased ErbB-2/Grb-2 interactions and enhanced MAPK activation (Janes *et al.*, 1997).

F. Tuning of ErbB Signaling by Receptor Endocytosis

The funneling of ErbB signaling into common pathways suggests that the variability in biological outcome would depend, at least partially, on kinetic regulation. A key determinant of signaling duration is the endocytic ligand-induced removal of receptors from the cell surface, a process called *downregulation*. Dependent on ligand binding, endocytosis and subsequent sorting to recycling back to the cell surface or degradation in lysosomes critically preserve a fine balance mediating activation by growth factors (Wells *et al.*, 1990). Indeed, alternative intracellular routing of ErbB proteins contributes to the diversification of signal transduction (Baulida *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996b). Whereas ErbB-3 undergoes slow endocytosis followed by recycling, most EGF-stimulated ErbB-1 molecules are destined to lysosomal degradation, the difference being expressed in the mitogenic potency of the complexes (Waterman *et al.*, 1998). The importance of receptor-surface expression is further assigned by the identity of the binding ligand as demonstrated in certain cells by TGF- α , a stronger activator than EGF, with an impaired ability to induce receptor downregulation (Ebner and Derynck, 1991). Interestingly, coexpression of ErbB-2 potentiates EGF signaling to the level achieved by TGF- α , following heterodimer disintegration in the early endosome, and receptor recycling to the cell surface (Lenferink *et al.*, 1998). Apparently, two processes determine intracellular sorting of endocytosed ErbB molecules (Fig. 4): First, recruitment of the c-Cbl adaptor protein positively shunts ligand-receptor complexes to degradation in lysosomes by elevating ubiquitination of the tyrosine-phosphorylated receptor (Levkowitz *et al.*, 1998). Second, once in the sorting endosome, some ligand-receptor complexes dissociate under the mildly acidic pH of this vesicular compartment, leading to uncoupling from c-Cbl and destination to the default pathway, namely, recycling. By contrast, complexes that resist low pH, such as the EGF/ErbB-1 complex, do recruit c-Cbl, undergo ubiquitination, and thereby they are destined to degradation in lysosomes.

In summary, signal transduction by the many EGF-like ligands is best described in terms of a neural-like network (see Fig. 5). This layered organization of multiple ligands, 10 dimeric receptors, and many downstream effector molecules funnels extracellular signals primarily into the Ras-Raf-MAPK pathway. However, despite its uniformity, the output of the network is variable: Prolonged activation is mediated by ErbB-2-containing heterodimers and may lead to transformation. Homodimers elicit only weak signaling because their endocytosis is relatively rapid, or because their selection of signaling molecules determines only transient coupling to the MAPK pathway. The network can be constitutively active if high ligand concentration is maintained through an autocrine or paracrine loop, or one of the receptors is mu-

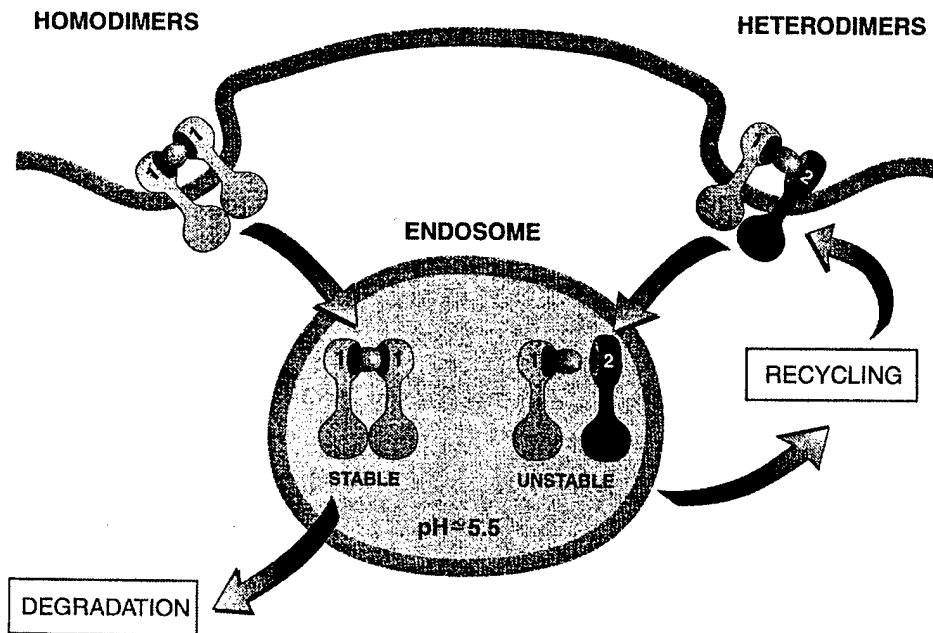


Fig. 4 Cell-surface receptor determination by endocytic routing. The identity of receptors paired by ligand binding determines the fate of the complex by intracellular routing. Following dimerization, receptors are aggregated within coated pits and internalized. The decision between degradation and recycling back to the cell surface is dependent on the stability of the complexes within cellular compartment. The acidic environment of the early endosome challenges the volatile interaction between the two receptors and the ligand causing the disintegration of unstable interactions. A heterodimer of ErbB-2 and ErbB-1 is readily dissociated at pH 5.5, enabling the rapid reappearance of the receptors on the cell surface. Such recycling reintroduces these molecules into the signaling milieu, thus conferring a sustained activation. ErbB-1 homodimers exhibit high stability under acidic conditions; remaining intact they are directed to subsequent compartments in which they undergo protein degradation. This results in a significant reduction in cell-surface expression influencing the kinetics of signaling.

tated or overexpressed. The latter mode of activation is most relevant to ErbB-2 because it allows biased formation of the most active receptor combinations—those containing ErbB-2.

VI. ErbB-DIRECTED CANCER THERAPY

The apparent correlation between ErbB expression and human cancer has attracted attention to these molecules as potential targets for the development of therapeutic modalities. Being mostly correlated to aggressiveness

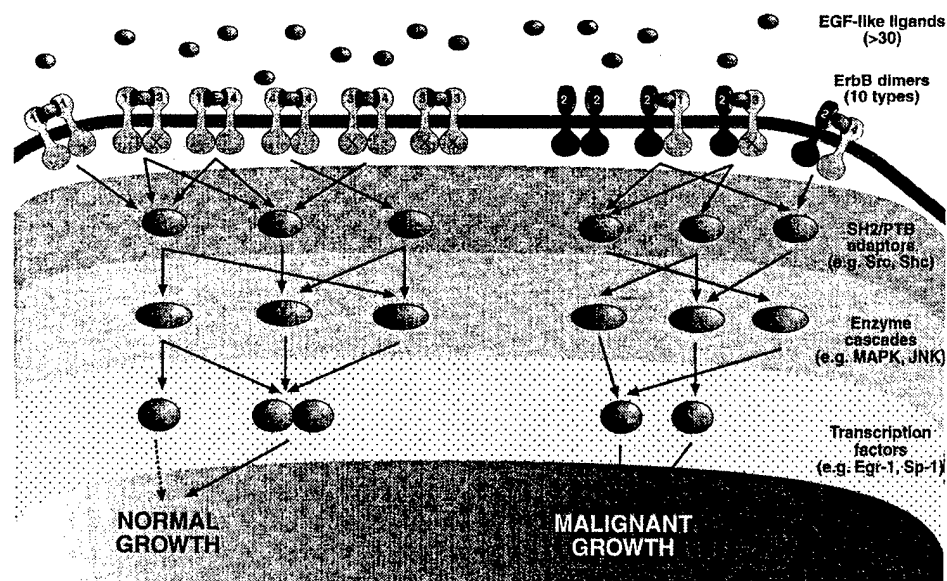


Fig. 5 The ErbB signaling network. Growth factor induced signaling is illustrated as protruding several consecutive layers. Multiple EGF-like ligands (>30) bind ErbB receptors recruited by differential and partially overlapping specificities, transmitting the signal through the membranal barrier. Ligand-receptor binding gives rise to 10 possible dimeric complexes, comprising the second level of signaling complexity. The identity of dimer-receptors is dependent on receptor expression and ligand affinity and results in the activation of a variety of downstream pathways. Effector molecules containing SH2 or PTB domains serve as adaptors by binding activated receptors and conveying the signal further, activating parallel, interacting, or overlapping cascades. The most frequently evoked is the activation of MAPK followed by its translocation to the nucleus and subsequent stimulation of transcriptional activity. Stimuli funneling through this pathway can lead to both differentiation and proliferation, promoting normal or pathological cell growth. This network pattern suggests that the absorbance of a wide variety of growth-stimulating signals results in the activation of a multitude of intracellular molecules, only to converge into few mainstream pathways. Complexity of such a system is contemplated to confer flexibility against fluctuations in extracellular conditions, ensuring an intact cellular response.

and poor prognosis of epithelial cancers, ErbB-2 has been assigned most of the attempts, utilizing strategies directed to inhibit its activity as well as promote specific immunity (Disis and Cheever, 1998a,b).

A. Immunotherapy

Antibodies that can block the biological activities of growth factor receptors are expected to alter the autocrine and paracrine loops. The rationale that specific ligand binding inhibition can decrease the mitogenic signaling

of ErbB receptors elicited the development of several antibodies, adequate and beneficial for human treatment (Fan and Mendelsohn, 1998). Antibody 225, a mouse monoclonal antibody that has been chimerized for use in humans, has a significant antitumor activity on a variety of cultured and xenografted cancer cell lines (Prewett *et al.*, 1996). It has been shown to successfully target primary lung cancers and metastasis (Divgi *et al.*, 1991), and is apparently well tolerated in patients receiving repeated administrations. Other ErbB-1-specific monoclonal antibodies have been assessed in phase I clinical studies for their safety and efficient binding in patients suffering from malignant gliomas (Faillot *et al.*, 1996), non-small cell cancer of the lung (Perez Soler *et al.*, 1994), and head and neck cancers (Modjahedi *et al.*, 1996). Likewise, successful inhibition of tumor growth has been accomplished by the use of monoclonal antibodies that specifically recognize ErbB-2, in either a conventional athymic murine system or in a transgenic animal model of breast cancer (Katsumata *et al.*, 1995). The immunological approach has recently been extended to patients: A phase II clinical trial revealed that a humanized antibody was clinically active in patients with ErbB-2-overexpressing metastatic breast cancers (Baselga *et al.*, 1996). Potentially, soluble ErbB-2 may interfere with the antitumorigenic effect of certain monoclonal antibodies directed to this receptor, and thus may limit the effect of immunotherapy (Brodowicz *et al.*, 1997). In addition, possible mechanisms underlying the antitumorigenic effect are constantly challenged. Different antibodies directed against the extracellular domain of the receptor have been shown to both decrease and increase receptor phosphorylation (Stancovski *et al.*, 1991), implying that simple inhibition of an evoked signaling cascade cannot explain the outcome. More appealing is the capability of inhibitory antibodies to downregulate the receptor from the cell surface (Hurwitz *et al.*, 1995) or, alternatively, to destabilize heterodimeric complexes (Klapper *et al.*, 1997), both mechanisms leading to a decreased signaling capacity. Anti-ErbB-2 antibodies have also been shown to affect the progression of the cell cycle either by inducing differentiation (Bacus *et al.*, 1992b) or by driving cells toward apoptosis (Kita *et al.*, 1996).

Patients with ErbB-2-positive cancers have been occasionally shown to develop an immune response against the protein (Disis *et al.*, 1994, 1997; Fisk *et al.*, 1995) predicting that antireceptor vaccines could be successful in evoking an anticancer response. The high expression of ErbB-2 on cancer cells, in comparison to normal tissues, suggests that such a response should be preferentially directed against malignancy with no or residual autoimmune toxicity. Originally, murine tumors overexpressing the rat oncogenic *neu* were successfully targeted and treated by immunization with a vaccinia virus recombinant of the protein's extracellular domain (Bernards *et al.*, 1987). Peptides from both intracellular (Ioannides *et al.*, 1992; Peiper *et al.*, 1997) and extracellular (Fisk *et al.*, 1995) portions of the receptor can elicit a spe-

cific response of cytotoxic T lymphocytes (CTL) originating from cancer patients. Tolerance to self-proteins has been suggested to depend on dominant epitopes allowing the promotion of an immune response to such molecules by the exposure of subdominant epitopes (Sercarz *et al.*, 1993). Accordingly, immunizing rats with peptides derived from the self-rat Neu, but not with the whole protein, can promote antibody and T-cell responses against the native protein (Disis *et al.*, 1996). Similar peptides, derived from the murine ErbB-2, can induce CTL activity, resulting in the suppression of growth of receptor-overexpressing cells in syngeneic hosts (Nagata *et al.*, 1997).

To confer ErbB-2 recognition to T cells, without the need for antigen processing while circumventing MHC restriction, expression of chimeric antibodies against ErbB-2 fused to the signaling subunit of the T-cell receptor was designed (Stancovski *et al.*, 1993). Adoptive transfer of such CTLs can markedly inhibit the growth of ErbB-2-transformed cells in nude mice (Moritz *et al.*, 1994) and in a syngeneic immunocompetent model (Alteneschmidt *et al.*, 1997a). ErbB-2-specific targeting and activation of T cells can also be achieved by fusing an antibody specific for ErbB-2 to a sequence encoding the extracellular domain of the B7-1 (Challita Eid *et al.*, 1998) or B7-2 (Gerstmayer *et al.*, 1997) T-cell costimulatory proteins. A similar methodology is utilized to attract and activate an additional arm of the immunological response including monocytes and macrophages. A bispecific antibody, directed against ErbB-2 and the Fc γ RIII, systemically administered to SCID mice bearing ovarian cancer significantly improved survival while associated with no observed toxicity (Weiner *et al.*, 1993). This encouraged a phase I clinical trial and contemplated future studies (Weiner *et al.*, 1995). Similarly, antibodies directed against Fc γ RI and ErbB-2 or ErbB-1 were evaluated in phase II clinical trials for treatment of a variety of neoplasms (Curnow, 1997; van-Ojik *et al.*, 1997) showing a promising range of responses as expressed by a reduction in metastasis and serum markers.

B. Gene Therapy

Transcriptional upregulation significantly contributes to ErbB-2 overexpression in cancers (Kraus *et al.*, 1987; Miller *et al.*, 1994; Pasleau *et al.*, 1993), suggesting that manipulation of the promoter activity can be utilized for therapy. Such is the selective expression of suicide genes driven by regulatory regions of the *erbB-2* promoter rendering cells sensitive to gancyclovir (Ring *et al.*, 1997). A different approach is the use of adenovirus type 5 early region 1A gene product (E1A) to repress ErbB-2 expression, suppressing the tumorigenic potential of overexpressing cells (Yu and Hung, 1991). The growth of human cancer cells of breast (J. Y. Chang *et al.*, 1997), ovary, and lung (Chang *et al.*, 1996) origin in nude mice is efficiently inhibited by the

viral product when delivered by vector or liposomes. This can also be effectively used to sensitize cells toward chemotherapy as demonstrated for breast cancer cells exposed to taxol (Ueno *et al.*, 1997). Alternatively, mRNA levels can be manipulated by conditional depletion; anti-ErbB-2 targeted hammerhead ribozymes, expressed under the control of a tetracycline-regulated promoter, can almost completely abrogate expression of the protein at the cell surface, resulting in the inhibition of tumor growth in nude mice, as well as in tumor regression upon tetracycline withdrawal (Juhl *et al.*, 1997). Similarly, antisense cDNA constructs encompassing different regions of the *erbB-2* gene inhibit the tumorigenicity of lung adenocarcinoma cells (Casalini *et al.*, 1997). Last, DNA delivery by adenoviral vectors has also been utilized for the introduction of an anti-ErbB-2 single chain antibody capable of retaining the protein within the cell. Intraperitoneal injection of the vector resulted in the reduction of tumor burden in SCID mice (Deshane *et al.*, 1997), encouraging a phase I clinical trial with ovarian cancer patients (Alvarez and Curiel, 1997).

C. Other Modes of Therapy

1. ANTIBODY-DRUG COMBINATION

As discussed earlier, tumors overexpressing ErbB-2 show lower responsiveness to adjuvant therapy that includes cyclophosphamide, methotrexate, and 5'-fluorouracil (CMF) (Allred *et al.*, 1992b; Gusterson *et al.*, 1992). Furthermore, ErbB-2 seems to synergize with the multidrug-resistant protein, p170mdr-1, rendering breast cancer cells more resistant to taxol (Yu *et al.*, 1998). One possible explanation for this sensitization could be the enhancement of cellular proliferation enabling cells surviving a therapeutic course to rapidly propagate (Pegram *et al.*, 1997). The increase in resistance to therapy conferred by receptor overexpression suggests that interference with ErbB-2 expression at the cell surface could lead to a better response (Torre *et al.*, 1997). Several studies have examined the possible advantage of combining anti-ErbB-2 effects with chemotherapeutic treatment. A synergistic inhibitory effect between mAbs to the EGF receptor and the DNA-damaging drug cisplatin has previously been reported (Aboud Pirak *et al.*, 1988). Similarly, an enhanced cytotoxicity of cisplatin, in breast and ovarian cells overexpressing ErbB-2, has been observed when cells were concomitantly exposed to an anti-ErbB-2 antibody (Hancock *et al.*, 1991; Pietras *et al.*, 1994). Further analysis of this phenomenon showed a reduction in both DNA synthesis and repair of cisplatin-DNA adducts in the presence of the antibody (Arteaga *et al.*, 1994; Pietras *et al.*, 1994), suggesting an elevated chemosensitivity as a result of antibody treatment. Enhanced cisplatin sensitivity in

the presence of anti-ErbB-2 mAbs, has been shown to depend on agonistic properties of the antibody (Arteaga *et al.*, 1994). Tyrphostin 50864-2, a low molecular weight tyrosine kinase inhibitor, can abrogate the elevated drug-mediated cell killing induced by an anti-ErbB-2 antibody. Moreover, the enhancement was not observed with an anti-ErbB-2 mAb that does not induce cell signaling (Arteaga *et al.*, 1994). A similar sensitization was achieved for the treatment with the anti-estrogen drug, tamoxifen (Witters *et al.*, 1997), as well as with TNF (Hudziak *et al.*, 1989), showing an enhanced inhibitory effect *in vitro* in the presence of an anti-ErbB-2 antibody.

2. IMMUNOTOXINS

Antibodies directed against ErbB-2 have been suggested as useful vehicles for the targeting of therapeutic agents to tumors. This approach is attractive due to both the correlation of receptor expression with cancer as well as the ability of antibodies to internalize with the receptor and introduce the toxic agent into the cell (Hudziak *et al.*, 1989; Hurwitz *et al.*, 1995; van Leeuwen *et al.*, 1990). Conjugates of mAbs and toxins have been used in preclinical trials as antitumor agents (Pastan and FitzGerald, 1991). Several immunotoxins have been constructed using various anti-ErbB-2 antibodies that have been coupled to Lys-PE40, a recombinant form of *Pseudomonas* exotoxin lacking its cell-binding domain (Batra *et al.*, 1992). Anti-ErbB-2-exotoxin successfully inhibits the growth of Schwannoma cells in nude mice (Alteneschmidt *et al.*, 1997b). Several other agents have been similarly targeted, including ricin (Rodriguez *et al.*, 1993), doxorubicin (Park *et al.*, 1995), and enzyme prodrugs (Eccles *et al.*, 1994), all presenting specific cell inhibitory effects. Ligands directed against ErbB proteins have also been examined as beneficial carriers, utilizing their high binding affinity to respective receptors. A fusion toxin of NRG1 with exotoxin-a induced complete regression of human breast cancer xenografts in nude mice (Groner *et al.*, 1997). Betacellulin-pseudomonas toxin fusion is effective against cells expressing ErbB-1 but not cells expressing ErbB-4, probably due to a limited internalizing capacity (Mixan *et al.*, 1998). A bispecific toxin combining the recognition ability of an anti-ErbB-2 antibody with that of TGF- α inhibits the growth of breast cancer cells *in vivo* (Schmidt *et al.*, 1996), probably by the induction of heterodimeric complexes and their subsequent internalization.

Drug delivery has also been attempted by antibody-targeting of drug-loaded liposomes. Immunoliposomes efficiently bind to cancer cells, delivering cytotoxic doses of doxorubicin in a targeted manner (Park *et al.*, 1995) as a function of their ability to internalize (Goren *et al.*, 1996). A prolonged tumor-localized supply of an ErbB-2 specific toxin has been elegantly achieved by the development of a new class of tumor-specific killer lymphocytes. These cells produce and secrete an antibody-targeted toxin in the vicinity of

the tumor, overcoming depletion by clearance, and result in high cytotoxicity toward tumors in an athymic murine model (Chen *et al.*, 1997).

3. TYROSINE KINASE INHIBITORS

In an attempt to inhibit the mitogenic signaling of receptor tyrosine kinases, several chemical compounds have been designed and synthesized to interfere with enzymatic activity (Klohs *et al.*, 1997). Two groups of molecules, termed *tyrphostins*, have been developed to bear selective specificities toward the ATP-binding sites of ErbB-1 or ErbB-2, resulting in an inhibition of proliferation of cells expressing the respective receptors (Osherov *et al.*, 1993). Tyrphostins specific for the ErbB-1 receptor inhibit primary glioblastoma cells from invading brain aggregates (Penar *et al.*, 1997) and prostate cancer from proliferation (Kondapaka and Reddy, 1996). A similar compound, capable of inhibiting activation of ErbB receptors, is a potent *in vivo* inhibitor of various human xenografts expressing ErbBs (Rewcastle *et al.*, 1998). AG825, a specific inhibitor of the ErbB-2 tyrosine kinase, sensitizes receptor-overexpressing cells to chemotherapy including doxorubicin, etoposide, and cisplatin (Tsai *et al.*, 1996), suggesting the involvement of ErbB-2 signaling in resistance toward chemotherapy. Thus, low molecular weight compounds capable of selective inhibition of the catalytic activity of specific ErbB proteins, either alone or in combination with other drugs, are potential future cancer therapeutic agents.

VII. CONCLUSIONS

Mounting experimental evidence now supports the notion that signaling by the ErbB receptors and their ligand growth factors may be explained in terms of a protein network. The signaling module evolved to function in inductive morphogenesis, but it is opportunistically exploited by malignant processes. This may not be limited to ErbBs; other growth factors, such as the hepatocyte growth factor and its sibling factors affecting cell migration and metastasis, and the vascular growth factors, which control angiogenesis, probably operate through similar signaling networks. It is currently unclear to which extent ErbB signaling is involved in the control of cell migration, angiogenesis, or apoptosis. For example, the possibility that ErbB proteins confer resistance to apoptosis induced by certain chemotherapeutic drugs is attractive but needs additional experimental support. Nevertheless, *in vitro*, as well as *in vivo* and clinical lines of evidence indicate that the major function of the ErbB network is to control the decision to proliferate or differentiate. Biochemically, this decision is executed by a linear cascade that

includes Ras, Raf, and MAPK. However, ErbB signaling recruits several other pathways, for example, the phosphatidylinositol 3'-kinase pathway, whose physiological role is less understood.

Crucial to understanding ErbB signaling is the unsolved biochemical role of the ligand-less oncoprotein, ErbB-2. The emerging notion arguing that ErbB-2 acts solely as a low-affinity/broad-specificity subunit of the three other ErbB receptors is appealing, because it attributes the oncogenic superiority of ErbB-2 to its probable ability to augment the proliferative actions of multiple stroma-derived growth factors. Accordingly, overexpression of ErbB-2 in tumor cells biases the formation of the respective heterodimer, thereby favoring formation of signaling complexes whose activity is more potent and prolonged. While this model explains the flexibility and robustness of the ErbB network, it also offers a scenario for therapy directed at blocking ErbB function in cancer cells: The lack of signaling autonomy of ErbB-2 means that not only antibodies to this protein, but also antagonists of specific growth factors, and blockers of other receptors and their enzymatic activities, will inhibit ErbB-mediated transformation. It is likely that specific ligands and certain heterodimeric ErbBs are more critical than others in tumors of different origins. Characterization of this specificity and its pharmacological targeting remain future challenges.

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